The Molecular Epidemiology of AmpC-Mediated Resistance in *Escherichia coli*: a study of clinical strains isolated from the South West region

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Abstract

Escherichia coli is a common human pathogen, capable of causing a wide range of infections. Some strains produce AmpC beta-lactamase, an enzyme able to affect the action of antibiotics. These strains are often resistant to cephalosporin antibiotics, causing problems for clinical treatment. The production of the enzyme can be mediated by either mutations in the chromosomal *ampC* promoter region, or through the acquisition of *ampC* plasmid genes from other species.

In this study, SYBR Green real-time PCR was used to detect bla_{AmpC} and $bla_{\text{CTX-M}}$ genes in *E. coli* isolates collected in Gloucester and four other laboratories in the South West region (Dorchester, Swindon, Taunton and Truro). Following a small pilot study, a larger study of 276 isolates collected from the five laboratories was undertaken. Isolates were tested for the presence of acquired bla_{AmpC} genes and $bla_{\text{CTX-M}}$ extended-spectrum beta-lactamase (ESBL) genes. The *ampC* promoter region for each isolate was sequenced, and all isolates were typed using multi-locus sequence typing (MLST).

AmpC plasmid genes were identified in 19 isolates (15 with CIT-type and 4 with DHA-type). The most common *ampC* promoter mutation was a T to A transition at position -32, seen in a total of 36 isolates. This was in contrast to other studies, in which the most common mutation is found at position -42. MLST confirmed the expected dominance of the ST131 clone (43.6% of isolates) within the cefpodoxime-resistant isolates in the South West. There was no evidence for a dominant clone within the AmpC plasmid-carrying isolates, but some potential evidence for a dominant clone of ST12 with the -32 *ampC* mutation present.

Overall, in 276 *E. coli* isolates with cefpodoxime-resistance, 25.8% were confirmed as having a genotype associated with AmpC resistance. The remaining isolates were largely carrying a $bla_{\text{CTX-M}}$ gene (57.1%) or another type of ESBL (16.4%).

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List of Abbreviations

bla	Beta-lactamase
BLAST	Basic Local Alignment Search Tool
bp	Base pair
CCG	Clinical Commissioning Group
CPD	Cefpodoxime
CT	Cycle Threshold
CTX	Cefotaxime
CXM	Cefuroxime
DI	Diversity Index
DNA	Deoxyribonucleic Acid
EDTA	Ethylene diamine tetra acetic acid
ESBL	Extended Spectrum Beta-Lactamases
FEP	Cefepime
FOX	Cefoxitin
MLST	Multi-Locus Sequence Typing
NCTC	National Collection of Type Cultures
NRES	National Research Ethics Service
PCR	Polymerase chain reaction
PFGE	Pulsed Field Gel Electrophoresis
PHE	Public Health England
RNA	Ribonucleic Acid
SNP	Single Nucleotide Polymorphism
ST	Sequence Type
TM	Melting Temperature

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1 Introduction

Escherichia coli is a common bacterium that has, over time, developed into a successful human pathogen. Not only a common component of the normal intestinal flora in humans, it is capable of causing a wide range of extra-intestinal infections; from urinary tract infections to septicaemia and meningitis (Jaureguy *et al.*, 2008). The impact of *E. coli* urinary tract infections alone globally is substantial. It is estimated that there are 130-175 million cases of uncomplicated cystitis in women, and 5.4 million cases of uncomplicated pyelonephritis each year worldwide. *E. coli* will be reported as the causative agent in 85-90% of these cases (Russo & Johnson, 2003), and other estimates suggest that half of all women in the United States will have a urinary tract infection at least once by the fourth decade of life (Foxman, 2002).

E. coli is also the most common cause of bacteraemia reported in the UK, with a total of over 35,000 cases in England and Wales during 2014 (Public Health England, 2015c). Mortality rates in these cases have been estimated to be in the region of 15%; equating to 4,800 deaths annually (Davies, 2013), therefore making options for effective treatment essential.

E. coli infections are usually easily treated with antibiotics. Some strains, however, produce beta-lactamase enzymes that inhibit the action of certain antibiotics. This can result in infections that are more difficult to treat, and which can be associated with a higher mortality rate (de Kraker *et al.*, 2011).

The beta-lactam group of antibiotics include the penicillins and cephalosporins, and represents the largest overall group of antimicrobials (Livermore & Woodford, 2006). These antimicrobial agents contain a beta-lactam ring as an integral part of their structure, and target the synthesis of the bacterial cell wall as their mode of action. Beta-lactamase enzymes produced by bacteria break down the beta-lactam ring, rendering the antibiotic ineffective.

The beta-lactamase enzymes represent a large and diverse collection of enzymes, including the extended spectrum beta-lactamases (ESBL) and AmpC beta-

lactamases. Both types of enzyme confer resistance to the cephalosporin antibiotics. ESBL enzymes are characterised by a broad pattern of resistance to cephalosporin antibiotics that has expanded to include the third-generation antibiotics (e.g. cefotaxime) (Pfaller & Segreti, 2006). Phenotypic detection in the laboratory is possible by demonstrating reversal of resistance using an inhibitor compound such as clavulanic acid. Clinical laboratories in the UK are recommended to screen all significant *E. coli* isolates for the presence of ESBLs using the third-generation cephalosporin cefpodoxime (Andrews & Howe, 2011).

The main difference between AmpC and ESBL enzymes is a lack of activity in the AmpC enzymes against fourth-generation cephalosporins (e.g. cefepime) and a failure to have the resistance reversed using clavulanic acid (Woodford *et al.* 2007). The presence of an AmpC enzyme, however, can still be confirmed phenotypically using an alternative inhibitor compound, such as cloxacillin (Babini & Livermore, 2000) or boronic acid (Coudron, 2005).

1.1 Extended-Spectrum Beta-Lactamases (ESBL)

The first beta-lactamases with an extended spectrum of activity were mutations of the TEM-1 and SHV-1 enzymes; commonly found as native enzymes in Enterobacteriaceae. Minor amino acid substitutions were sufficient to expand the hydrolyzing activity beyond the penicillins (Cantón *et al.*, 2008). These new enzymes (e.g. SHV-2 and SHV-5) were called extended-spectrum beta-lactamases due to their ability to hydrolyze the third-generation cephalosporins, but not the cephamycins. Over the last decade, there has been a significant shift in the prevalence of different ESBL types, with the TEM and SHV ESBL enzymes being largely replaced by CTX-M enzymes (Livermore *et al.*, 2007).

Although the CTX-M enzymes were first discovered in 1989 (Bauernfeind, Grimm & Schweighart, 1990), they did not reach prominence until early in the 21st century. The first CTX-M enzyme was detected in an *E. coli* strain isolated from an ear in a 4-month old child with otitis media. The CTX refers to the enhanced activity against cefotaxime and the –M refers to Munich, the city of first isolation. Phylogenetic

analysis indicates that the CTX-M enzymes originated from chromosomal genes from various *Kluyvera* spp. (Cantón *et al.*, 2008).

In the UK, CTX-M genes were first reported in 2000 (Alobwede *et al.*, 2003), but by 2004 were the predominant cause of cephalosporin resistance in *E. coli* and *Klebsiella pneumoniae* (Potz *et al.*, 2006). A later study of *E. coli* bacteraemia isolates in Leeds between 2010 and 2012 found 68% of ESBL-producing isolates were carrying CTX-M Group 1 genes (Horner *et al.*, 2013).

The CTX-M enzymes are characterised by enhanced activity against cefotaxime, and are classified into five main groups according to amino acid sequence similarities. The Group 1 enzymes include CTX-M-1, -3, -10, -12 and CTX-M-15. Group 2 enzymes include CTX-M-2, -4, -5, -6, -7 and -20. Group 8 includes CTX-M-8. Group 9 enzymes include CTX-M-9, -13, -14, -16, -17, -19, -21 and -27. Group 25 includes the CTX-M-25 and CTX-M-26 enzymes (Bonnet, 2004).

There are >170 different recorded variants of CTX-M enzymes (http://www.lahey.org/studies) (last checked 02/10/15). The most common are CTX-M-14 and CTX-M-15; found in human, animal and environmental sources worldwide (Cantón *et al.*, 2008). The predominant reason for the increase in the prevalence of CTX-M ESBL enzymes is the global dissemination of the ST131 pandemic *E. coli* clone that carries the gene for CTX-M-15 production (Coque *et al.*, 2008) (See Section 1.5).

1.2 AmpC Beta-Lactamases

E. coli strains produce AmpC beta-lactamase enzymes using two separate mechanisms. Firstly, all strains have genes encoded in the chromosome for enzyme production (Olsson *et al.*, 1983). The level of production is tightly controlled and at a level so low as to not cause detectable resistance. Mutations can occur in the *ampC* promoter region that affect the level of control, leading to hyper-production of the AmpC enzyme and potential treatment failures (Caroff *et al.*, 2000).

E. coli strains can also acquire the genetic material coding for AmpC enzyme production from other species. Plasmids carrying *ampC* genes are derived from species such as *Enterobacter* sp. and *Citrobacter* sp.

1.2.1 Plasmid-Mediated AmpC Resistance

Although there is some evidence of AmpC-carrying plasmids as early as 1976 (Bobrowski *et al.*, 1976), the first credible report was in 1989 in the United States, when Bauernfeind *et al*. (1989) reported the isolation of a *K. pneumoniae* strain from a patient's wound site. This first plasmid gene was called CMY-1, after its cephamycinase activity. Later, Papanicolaou *et al*. (1990) identified a plasmid gene named as MIR-1, with the inhibition profile indicative of a beta-lactamase enzyme. Sequencing a short (150bp) region found 90% identity with the *ampC* chromosomal gene of *Enterobacter cloacae*, but only 71% identity with the *ampC* gene in *E. coli*.

The first reported UK case was in 1989 (Woodford *et al.*, 1990), in a patient originating from Pakistan. The plasmid was isolated from an *E. coli* strain and was subsequently shown to transfer resistance to three different genera (Payne, Woodford & Amyes, 1992). The plasmid was originally named BIL-1, but with the advent of more sophisticated sequencing methods has been confirmed to be identical to the CMY-2 plasmid gene (Barlow & Hall, 2002). By 2009, CMY-2 was the most commonly reported AmpC plasmid gene worldwide (Jacoby, 2009).

In 2002, Perez-Perez and Hanson published a set of primers for detecting and classifying the AmpC plasmid genes into six groups (Perez-Perez & Hanson, 2002). Using traditional end-point PCR methods, with a multiplexed primer set, isolates could be placed into one of six groups: ACC, FOX, MOX, DHA, CIT and EBC. The six groups were based on the sequences derived from similar species: *Hafnia alvei* (ACC), *Morganella morganii* (DHA), *Citrobacter freundii* (CIT) and *Enterobacter cloacae* (EBC).

CMY-2 (part of the CIT group) has a wide geographic spread, and is often reported as the most common AmpC plasmid-mediated gene in surveillance studies (European Centre for Disease Prevention and Control, 2014). The potential

reservoirs for the CMY-2 plasmid genes are numerous and widespread. Voets *et al*. (2013) screened retail chicken products in the Netherlands and compared resistant *E. coli* isolates with human isolates. In the chicken isolates, 12.2% carried a CMY-2 plasmid gene, compared to 5.2% of the human isolates. Overall, the study results indicated that 68% of human AmpC plasmid-carrying *E. coli* isolates have the same *ampC* plasmid gene as that found in poultry meat.

In a UK study of raw chicken products imported into the UK from South America, 42% of the 141 cefpodoxime-resistant *E. coli* isolates carried a CMY-type AmpC plasmid (Dhanji *et al.*, 2010). The majority of the other isolates had CTX-M-type ESBL genes, with two isolates shown to have both CMY-type and CTX-M-type genes.

Another Dutch study (Dierikx *et al.*, 2012) investigated the presence of antibiotic resistance genes in veterinary isolates of Enterobacteriaceae; isolated from dogs, cats, horses and a turtle. Most of the isolates came from cases of urinary tract infections in the animals. CMY-2 plasmid genes were found in 13.8% of the cephalosporin-resistant isolates, with other isolates mainly having CTX-M type genes present. The presence of bla_{AmpC} genes in cefoxitin-resistant *E. coli* strains isolated from water sources and beaches across Canada was investigated in 2009 (Mataseje *et al.*, 2009). CMY-2 AmpC plasmid genes were found in 77.5% isolates examined.

Plasmids carrying genes for AmpC beta-lactamases can also carry multiple other resistance genes, including those for resistance to the aminoglycosides, quinolones and other beta-lactamases (Jacoby, 2009). Also, the co-occurrence of multiple AmpC plasmid genes in the same strain has been reported in a number of species, including *E. coli* (Chérif *et al.*, 2015). Resistance genes can also occur alongside virulence genes. The large outbreak in Germany in 2011 of *E. coli* serotype O104 was found, after whole genome analysis, to be carrying genes for shiga-toxin production and also the CTX-M-15 gene (Rasko *et al.*, 2011). The outbreak had a reported total number of 4075 cases and 50 deaths.

1.2.2 Chromosomal AmpC Resistance in E. coli

The promoters for *E. coli* RNA polymerase have two highly conserved regions, located 10 and 35 base pairs upstream from the transcription start site. This is the case in a number of different promoters for the organism, including the *ampC* promoter. The two conserved sequences for the -10 and -35 regions are TATAAT and TTGACA, respectively (Hawley & McClure, 1983). In addition to the sequences of these regions, the actual distance between them is important for promoter strength, with a 17bp distance creating a stronger promoter. Promoters with distances of 15bp and 20bp have been reported to retain only partial function (Hawley & McClure, 1983). The -10 and -35 box regions are key to the success of the transcription process, and are responsible for the fixation of the sigma factor subunit of RNA polymerase (Busby & Ebright, 1994). The *ampC* promoter for *E. coli* is different by a single base pair in each of the two boxes, and has an inter-box distance of 16 base pairs (**Figure 1-1**). It is these small changes that reduce the power of the promoter and the associated level of enzyme production (Corvec *et al.*, 2007). Mutations that affect either of the two box regions, or the distance between them, can have an impact on promoter strength, and the corresponding level of enzyme production.

All *E. coli* strains have the chromosomal *ampC* gene encoding for AmpC beta-lactamase production (Hawley & McClure, 1983). In most cases, the gene is under tight regulation and is expressed constitutively in low amounts; rarely producing enough enzyme to cause clinical resistance. The potential role of the *ampC* gene in causing an increase in the production of beta-lactamase enzymes was first suggested in 1979 (Bergstrom & Normark, 1979), when six isolates of *E. coli* were investigated due to unusual resistance. The first report in a clinical isolate was in 1982 (Olsson, Bergstrom & Normark, 1982), when four single base pair mutations in the *ampC* promoter region were described that resulted in a 40-fold increase in AmpC enzyme production. One of the mutations was located near the -35 box region, at position -42. This mutation is the most frequently reported in studies, and creates a displaced -35 box with a sequence closer to that of the *E. coli*

consensus sequence (TTGACA) (**Figure 1-1b**) (Mulvey *et al.*, 2005; Mammeri *et al.*, 2008; Jorgensen *et al.*, 2010; Bogaerts *et al.*, 2010).

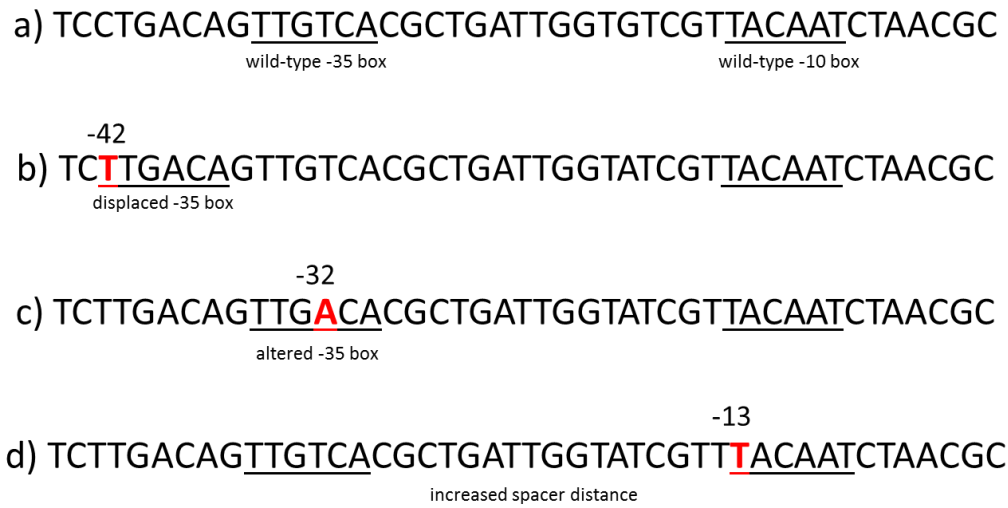


Figure 1-1: Section of the *ampC* promoter region in *E. coli*, with examples of mutations.
(a) The wild-type hexameric -10 and -35 boxes
(b) C->T transition at position -42 creates a displaced -35 box with a sequence identical to the *E. coli* standard promoter (TTGACA)
(c) T->A transition at position -32 creates an altered wild-type -35 box with a sequence identical to the *E. coli* standard promoter (TTGACA)
(d) Insertion of a single nucleotide at position -13 increases the spacer distance from 16bp to 17bp.
Numbering of positions is according to Jaurin *et al*. (1981).
Figure adapted with permission from Lewis *et al*. (2013).

Other mutations in the *ampC* promoter region have been reported to cause a significant increase in enzyme production (Caroff *et al.*, 2000). Mutations are usually grouped into those that create a displaced promoter region and those that change the existing promoter region. In addition to the -42 mutation (C to T), commonly reported mutations include a T to A transition at position -32 (**Figure 1-1c**) and a 1 or 2 bp insertion at position -13 (**Figure 1-1d**) (Tracz *et al.*, 2007).

The -32 mutation (T to A) was first described by Kobayashi *et al*. (1990), and has since been reported to increase enzyme expression by a factor of 46-fold (Tracz *et*

al., 2007). This mutation changes the existing -35 box region to one with a sequence closer to the *E. coli* consensus sequence.

The insertion of one or two bases in position -13 increases the distance between the -10 and -35 regions. The importance of the spacer distance between the -10 and -35 box regions in the *ampC* promoter was confirmed by Ayers *et al.* (1989). Moreover, Tracz *et al.* (2007) demonstrated that isolates with a single base pair insertion at position -13 had a 24- to 61-fold increase in enzyme expression.

The -42, -32 and -13ins mutations are often found together with mutations at other locations in the promoter region. In a large Canadian study, Mulvey *et al*. (2005) found mutations at 29 different locations in the 271bp sequenced *ampC* promoter region. In the 166 *E. coli* isolates investigated, a total of 49 different mutation patterns were found; all but one of which had multiple locations involved. Other mutations have been reported, including mutations in the attenuator and coding regions, but these have been shown to have a lesser impact on the level of enzyme production (Tracz *et al.*, 2007).

1.3 Epidemiology of AmpC-Mediated Resistance

European surveillance data indicate that the mean rate of resistance for third generation cephalosporins in invasive *E. coli* isolates (from blood and CSF) was 12.6% for the participating countries in 2013 (European Centre for Disease Prevention and Control, 2014). The individual country rates varied from 5.0% for Iceland to 39.6% for Bulgaria, with the UK having a rate of 14.7%. Overall, the adjusted population mean rate increased significantly from 9.5% to 12.6% over the four-year period up to 2013. Although some European data exist for ESBL prevalence rates, this does not include UK strains or the inclusion of data for the prevalence of AmpC-producing strains.

The incidence of AmpC-mediated resistance in most areas of the UK is unknown. Although a national surveillance programme exists for *E. coli* bacteraemia strains, this does not extend to characterising the mechanisms of resistance or to include other sample types (e.g. urine). Laboratory surveillance data submitted to Public

Health England (Public Health England, 2014) indicate that 10.9% of *E. coli* bacteraemia isolates in the UK were phenotypically determined as being resistant to cephalosporins during 2013. The percentage of cephalosporin-resistant cases was seen to vary in regions throughout the UK, with London and Cheshire, Warrington, Wirral having the highest at 15% and Devon, Cornwall having the lowest rate at 6%. This suggests that the types of resistance mechanisms found in *E. coli* may also vary between regions. There are no national data, however, for the actual presence of ESBL or AmpC resistance genes.

In 2006, Potz *et al*. (2006) tested 1253 cephalosporin-resistant Enterobacteriaceae isolates that had been submitted to the English national reference unit (Colindale, London). Of these, 16.9% were high-level AmpC producers. Another study reported in 2006 (Hopkins *et al.*, 2006a), investigated a different collection of *E. coli* isolates submitted to the national reference laboratory between 1995 and 2003. In one of the few studies to investigate *ampC* chromosomal mutations in the UK, 103 cephalosporin-resistant *E. coli* isolates were examined. A total of 37 isolates were confirmed as having possible AmpC genes, based on cefoxitin resistance, with 67.6% carrying an AmpC plasmid gene and 29.7% having chromosomal *ampC* mutations. However, as these isolates had been submitted to the national reference laboratory, they may not truly reflect the populations of strains circulating in clinical samples.

Woodford *et al*. (2007) investigated 135 *E. coli* isolates for the presence of AmpC plasmid genes. All isolates had been selected from those referred to the reference laboratory as having a phenotype suggestive of an AmpC plasmid-mediated resistance (cephalosporin resistance without potentiation by clavulanic acid). A total of 67 (49.6%) isolates were confirmed as carrying an AmpC plasmid gene, 59 of which were of the CIT group, 4 were of the ACC group, 3 were of the FOX group and 1 was from the DHA group.

Studies investigating the normal human carriage of *E. coli* strains with *ampC* chromosomal mutations are rare. Van Hoek *et al*. (2015b) investigated 1033 rectal swabs from volunteers in Denmark. In 65 *E. coli* isolates with cephalosporin-

resistance, 8 isolates (0.77% prevalence) had mutations in the *ampC* chromosomal promoter region. Four isolates had a -42 mutation, 3 isolates had a -32 mutation and one isolate had a nucleotide insertion at position -14.

1.4 Clinical Impact of Cephalosporin Resistance

There were over 35,000 reported cases of *E. coli* bacteraemia in England and Wales in 2014, with the incidence rate per 100,000 population increasing by 16.7% between 2010 and 2014 (Public Health England, 2015c). *E. coli* strains represent about one-third of all bacteraemia cases (Davies, 2013), and evidence published by de Kraker *et al.* (2011) suggests that patients with septicaemia due to a cephalosporin-resistant *E. coli* strain have a mortality rate of 30%, compared to 15% for patients with a susceptible strain. Other estimates of increased mortality vary, but most authors agree that there is an increase.

UK data for 2011 indicate that 45% of *E. coli* bacteraemia cases have a urinary tract infection as the primary source (Davies *et al.*, 2012). It is in these systemic cases that infection with a cephalosporin resistant strain is more significant, with length of hospital stay and mortality both frequently being reported as adversely affected. In a Spanish study, Peralta *et al*. (2007), reported the results of a retrospective study of 663 *E. coli* bacteraemia cases from 1997 to 2005. They demonstrated that patients with a multi-drug resistant strain were less likely to receive correct empirical treatment and had a higher mortality rate. The definition of a multi-drug resistant strain was phenotypic evidence of ESBL or AmpC production, with resistance to 3 or more antibiotics.

In a UK prospective study of *E. coli* bacteraemia cases (Melzer & Petersen, 2007), the proportion of patients who died within 30 days following onset of bacteraemia illness was significantly higher in the ESBL-producing isolates at 60.9%, compared to 23.7% for the non-ESBL isolates. This increased risk of death from an ESBL-producing *E. coli* infection was actually associated with the delay in appropriate treatment. Once the delay in appropriate treatment had been excluded from the dataset, the ESBL cases actually had a longer time to death (7 days instead of 5 days) and a shorter inpatient stay (9 days instead of 12 days). Rottier *et al*. (2012)

suggest that the outcomes of observation studies of this nature are highly susceptible to confounding factors, which may mislead investigators. Patients with an increased severity of illness will generally require a longer stay in hospital and more antibiotics for treatment. Moreover, the likelihood of colonisation remaining and subsequent infection happening is higher with resistant bacteria.

Not all studies have found a significant difference between ESBL-producing and non-ESBL-producing strains. In a retrospective study of five years' bacteraemia data in France, Denis *et al.* (2015) found that the presence of an ESBL-producing *E. coli* isolate was not associated with a longer stay in hospital; and although there was a higher mortality rate, it was not significant (30% vs 27%). Another study (Ofner-Agostini *et al.*, 2009) also failed to determine a significance difference in mortality rates between cephalosporin-resistant and susceptible isolates of *E. coli* and *K. pneumoniae* (13% vs 7%) in Canada.

The number of studies investigating the impact of AmpC-mediated resistance on the clinical outcome of *E. coli* infections is less common. Most studies have focussed on the impact of strains with an inducible AmpC enzyme, such as *Enterobacter* spp. (Kaye *et al.*, 2001; Choi *et al.*, 2008). Rand *et al.* (2011) investigated AmpC-producing Enterobacteriaceae in a case-control study against non-AmpC isolates. The presence of an AmpC enzyme was only determined using phenotypic methods, and the case group only included three isolates of *E. coli*. However, the study did identify a slightly higher 30-day mortality rate for the AmpC group (9% vs 6%), but this was not found to be significant. In a study not linked to bacteraemia cases, Sidjabat *et al*. (2009) determined in a retrospective cohort study that patients with infections caused by isolates with CMY-type enzymes were significantly more likely to have a symptomatic infection, when compared with ESBL-producing isolates.

1.5 *E. coli* ST131 is a Successful Global Strain

The dominance of CTX-M genes as the primary cause of ESBL-mediated resistance in *E. coli* is due to the global expansion of one particular clone, called ST131 after its multi-locus sequence type (MLST). The ST131 clone has spread extensively across the world since it was first reported in 2008 (Nicolas-Chanoine *et al.*, 2008), and has

been reported in multiple countries and continents (Peirano & Pitout, 2010), including the UK (Lau *et al.*, 2008; Gibreel *et al.*, 2012; Horner *et al.*, 2013). Studies on retrospective isolates in America and Canada suggest that the clone may have first appeared earlier, probably around 2003 (Johnson *et al.*, 2012; Peirano *et al.*, 2012). Although MLST was not in use at the time, a UK study in 2004 (Woodford *et al.*, 2004) described the likely presence of the ST131 clone, with 37.8% of CTX-M-producing isolates identified as a single clone using pulsed-field gel electrophoresis.

The reason for this degree of global spread is not clear. Possibilities include higher transmissibility, enhanced virulence or increased antibiotic resistance (Banerjee & Johnson, 2014). Transmissibility studies for *E. coli* ST131 have shown that cases of intra-familial spread can occur: between mother and daughter (Johnson *et al.*, 2010), between two sisters (Price *et al.*, 2013) and even between household pets (Johnson *et al.*, 2009).

The case for enhanced virulence is supported by the fact that ST131 strains are more common in cases of invasive disease (e.g. sepsis) than non-invasive disease. Alhashash *et al*. (2013) demonstrated that the prevalence of ST131 isolates in blood cultures was 21%, compared to 7% for urine cultures. Other studies have shown that the prevalence of ST131 isolates is higher in cases of pyelonephritis than routine cystitis and also when compared with healthy faecal carriage (Kudinha *et al.*, 2013b, 2013a). Banerjee *et al*. (2013) suggest that ST131 strains are more likely to cause recurrent or persistent urinary tract infections, and ST131 strains are also associated with a higher frequency of sepsis (Tchesnokova *et al.*, 2013).

Antibiotic resistance is a key feature of the ST131 clone, with strains accounting for 70-80% of all fluoroquinolone-resistant *E. coli* isolates and two-thirds of ESBL-producing isolates (Banerjee & Johnson, 2014). Banerjee *et al.* (2013) demonstrated that the prior use of a fluoroquinolone or third-generation cephalosporin antibiotic was significantly associated with the acquisition of a ST131 strain. Colpan *et al.* (2013) reported that ST131 isolates accounted for 78% of the fluoroquinolone-resistant isolates and 64% of the ESBL-producing isolates, but only 7% of fluoroquinolone-sensitive isolates, in a multi-centre study in the US.

1.6 Principal Methods Used in this Study

1.6.1 Real-time PCR / SYBR Green

This study made use of real-time SYBR Green PCR technology with melting curve analysis. The use of fluorescence to monitor the progress and outputs of PCR reactions was first described in 1992 (Higuchi *et al.*, 1992). This enabled the real-time detection of PCR products, without the need for a separate detection stage such as gel electrophoresis. It also meant that reactions could be contained within a single tube, removing the risk of environmental contamination with PCR amplicons. The use of real-time PCR in this study was driven by the availability of equipment. Many clinical microbiology laboratories have real-time PCR instruments for diagnostic purposes, some of which can be used for melting curve analysis (personal observation).

In 1997, the use of melting curve analysis was highlighted as a means to confirm the presence of intended PCR products and also to differentiate between products based on the melting points (Ririe, Rasmussen & Wittwer, 1997). The melting point of a particular PCR product is determined by its GC content and the length of the target sequence. As fluorescence is monitored continuously during a temperature cycle, the melting point is observed as a rapid loss of fluorescence at a particular temperature. The melting curves can be transformed into a graph of melting peaks using a first-derivative calculation (**Figure 1-2**). Assuming that the GC content of two sequences was the same, a 40bp product would melt at a temperature 12^{o}C lower than a 400bp product, thus allowing the presence of primer dimers to be identified (Ririe, Rasmussen & Wittwer, 1997). The presence of a melting peak in the expected melting temperature range is analogous to the presence of bands on gel electrophoresis (Giglio, Monis & Saint, 2003; Boot *et al.*, 2013).

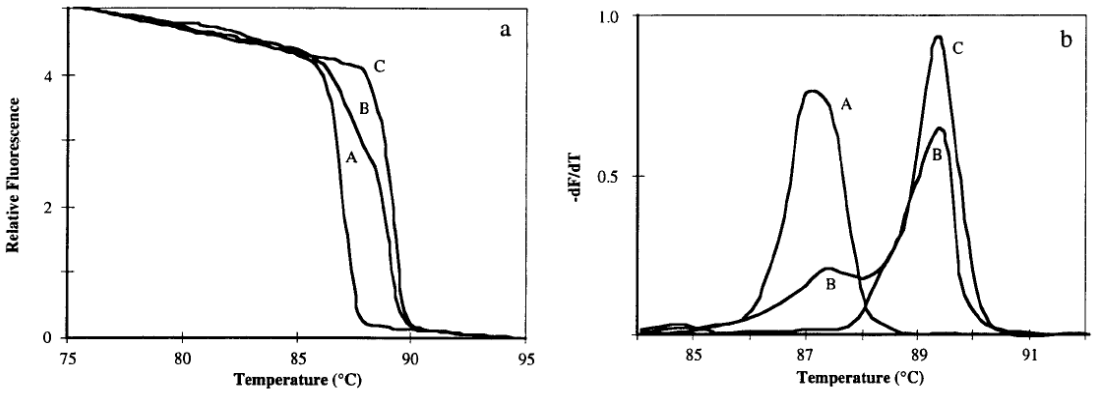


Figure 1-2: Real-time PCR melting curves (a) and melting peaks (b) for mixtures of amplification products.
Sample A represents a purified 180bp fragment from the Hepatitis B gene. Sample C represents a 536bp fragment from the human b-globin gene. Sample B is a mixture of Samples A and C. The melting curves were transformed into first derivative melting peaks. Reproduced with permission from Ririe *et al*. (1997).

Although early papers still used ethidium bromide as the fluorescent dye, this has since been replaced with less-hazardous chemicals such as SYBR Green (Wittwer *et al.*, 1997). SYBR Green is an intercalating dye that binds preferentially to double-stranded DNA. Its use in clinical diagnostic and research assays has been well reported, particularly for detecting virulence genes and antibiotic resistance genes in Enterobacteriaceae isolates. Mendes *et al*. (2007) used SYBR Green assay to detect the presence of metallo-beta-lactamase genes in Enterobacteriaceae isolates in Brazil. Guion *et al*. (2008) developed a SYBR Green assay to detect and characterise the presence of eight virulence genes in isolates of diarrhoeagenic *E. coli* in the US.

Chandramohan and Revell (2012) used a SYBR Green melting curve analysis to detect bla_{ESBL} genes in a range of Enterobacteriaceae isolates from paediatric patients in the US. Boot *et al.* (2013) demonstrated that a SYBR Green assay could be used to detect bla_{ESBL} genes in Enterobacteriaceae isolates. The assay was reported as having a sensitivity ten-fold higher using melting curve analysis when compared to gel electrophoresis.

Molecular methods for detecting AmpC plasmid genes have developed since the first publication in 2002 (Perez-Perez & Hanson, 2002). More recently, Brolund *et al*. (2010) reported using a SYBR Green real-time PCR method for detecting the six AmpC plasmid groups. The method also used melting curve analysis to differentiate the PCR products in multiplexed reactions. Geyer and Hanson (2014) have also reported using real-time PCR and melting curve analysis for the detection and differentiation of AmpC plasmid genes.

1.6.2 Multi-Locus Sequence Typing (MLST)

First described in 1998, multi-locus sequence typing is a method of typing bacteria using the sequences of housekeeping genes at a number of different loci (Maiden *et al.*, 1998). The first scheme published was for *Neisseria meningitidis*, in which six housekeeping genes were selected from a panel of eleven to create a typing method. The DNA sequence of each gene locus is determined and then assigned an allele profile number. The final sequence type (ST) number is derived from the profile of the six allele numbers. For example, the allelic profile of *N. meningitidis* ST1 is 1, 1, 1, 1, 3, 3 (Maiden *et al.*, 1998).

Maiden *et al.* (1998) describe the principal advantage of MLST as being the portability of the results, with data being truly comparable between laboratories. This is in contrast to typing methods such as pulsed-field gel electrophoresis, which rely on the interpretation of gels. Maiden *et al.* (1998) also suggested the creation of global databases for each species, so that data from MLST studies can be standardised to facilitate global epidemiology. This has subsequently been completed, with the creation of the PubMLST website, in which 75 MLST schemes are hosted for different bacterial species (www.pubmlst.org) (Jolley & Maiden, 2010).

Strains can be grouped into clonal complexes, based on sharing the same allele sequences at a number of the different loci. Clonal complexes are usually comprised of a single dominant sequence type, with a number of less common close relatives. Usually, the members of a complex will share identical allele sequences at all but one locus. The emergence of clonal complexes within a population occurs as a

particular sequence type increases in frequency and as a result of genetic changes over time becomes the dominant founder of the complex (Feil *et al.*, 2004).

There are two MLST schemes for *E. coli*. The first was described by Wirth *et al*. (2006), and is widely known as the Achtman scheme. In this scheme, seven housekeeping genes are used to generate the allelic profile: *adk* (adenylate kinase); *fumC* (fumarate hydratase); *gyrB* (DNA gyrase); *icd* (isocitrate dehydrogenase); *mdh* (malate dehydrogenase); *purA* (adenylosuccinate dehydrogenase) and *recA* (ATP/GTP binding motif). The use of housekeeping genes helps to ensure that the measured variation is neutral, as any nucleotide changes will be constrained by the need to retain essential biochemical functions (Spratt & Maiden, 1999).

The second scheme was developed at the Pasteur Institute in 2008 (Jaureguy *et al.*, 2008), and is known as the Pasteur method. The Pasteur method uses eight loci to generate the sequence type profile, one of which (*icdA*) is at the same locus as the Achtman scheme. The two schemes have broadly compatible results, although the Pasteur scheme has fewer registered sequence types and has been mainly used for investigating animal and environmental strains (Bae *et al.*, 2014)

Before the advent of whole-genome sequencing, pulsed-field gel electrophoresis (PFGE) was generally considered the gold standard for analysing outbreaks of infections (Padmanabhan *et al.*, 2013). A few studies have compared the resolving power of PFGE against that of MLST. Nemoy *et al.* (2005) investigated 40 ESBL-producing *E. coli* isolates, finding 19 different PFGE subtypes. One of the PFGE types was dominant, comprising 30% of isolates. This single PFGE type, however, was separated into five different sequence types using MLST. Overall the MLST scheme used in the study found 22 MLST sequence types, but it was a modified scheme using eight housekeeping genes supplemented with two antibiotic resistance genes. Although the MLST scheme was shown in this particular study to have a higher level of discrimination compared to the PFGE method, it is worth noting that this was only the case when the two antibiotic resistance genes were included in the allele profile. In general, however, PFGE is considered to be more discriminatory than MLST.

Tartof *et al*. (2005) used the Achtman MLST scheme to examine 45 *E. coli* isolates from human, animal and environmental sources. Of 19 isolates confirmed as being ST69 type by MLST, a total of 14 different subtypes were identified by PFGE. Another example of the difference between the two methods is provided by Gibreel *et al*. (2012), in which 8 different PFGE profiles were found in isolates of ST73 and 9 PFGE profiles were found in the ST131 isolates.

Although MLST may not be as good as other techniques at resolving outbreak situations, the fact that it can reveal relationships among isolates that appear to be different could also be considered a benefit. Woodford (2008) suggests that the ability to group isolates together that have previously been shown as distinct by other methods is important in the identification of potential epidemic clones. Highlighting potential relationships between strains can be an important initial step in identifying the need for interventions. It is the use of MLST that identified the presence of the global ST131 *E. coli* strain.

1.7 Study Design

1.7.1 Aim

The principal aim of this study was to determine the molecular characteristics of clinical *E. coli* isolates producing AmpC beta-lactamase enzymes in the South West of England.

1.7.2 Objectives

a) To confirm the utility of SYBR Green real-time PCR as a method for the detection of *bla* genes in *E. coli*
b) To determine whether a dominant AmpC-producing clone of *E. coli* exists within the South West population
c) To determine the genotypic characteristics of AmpC-producing *E. coli* isolates present in the South West population
d) To determine whether there is evidence that the *E. coli* populations differ between the five laboratories studied.

1.7.3 Ethics

The study was approved by the South West (Central Bristol) National Research Ethics Service (NRES) Committee, under reference 11/SW/0224. Local site approval was also obtained for the Gloucester laboratory, and for each of the other four participating laboratories.

1.7.4 Funding

The study was part-funded by two research grants from the Institute of Biomedical Science (IBMS).

2 Materials & Methods

2.1 Principal Equipment Used

The principal equipment used in this study is shown in **Table 2-1**.

Table 2-1: List of principal equipment used in the study, with manufacturer and model/version details

Equipment	Function	Model / Version	Manufacturer
Smart Cycler II	Real-Time PCR	DX 2.0	Cepheid, US
Geneious	DNA Analysis Software	7.1.7	Biomatters, NZ
Vortexer	Vortexer	SO 200	Labnet
Mini-Centrifuge	Centrifugation	Mikro 200	Hettich
Qubit 2.0	Fluorimeter	2.0	Invitrogen

2.2 Bacterial Control Strains

A set of six bacterial control strains was obtained from Public Health England (Colindale, UK), representing each of the six AmpC plasmid groups. The six control strains were those used by the PHE laboratory as positive controls for their in-house PCR assay for AmpC plasmid genes (Woodford, 2010 personal communication), but have not been registered with the National Collection for Type Cultures (NCTC). A further control strain was used as a positive control in the CTX-M ESBL PCR assays, and a wild type control strain known to lack bla_{AmpC} and bla_{ESBL} genes was used for comparison of promoter sequences. Details of all the control strains used in the study are given in **Table 2-2**.

2.3 SYBR Green PCR / General PCR Methods

2.3.1 Preparation of DNA Template

DNA templates were prepared using a mechanical lysis method (Woodford, 2010). Each control strain was inoculated onto a Columbia Horse Blood Agar plate (Oxoid, UK) and incubated overnight at 37^{o}C in air. A heavy bacterial suspension, equivalent to MacFarland Standard 4.0 (Biomerieux, UK), was prepared in 100µl water (PCR grade) in 1.5ml PCR tubes (Sarstedt, UK). Tubes were vortex-mixed for 2 minutes and then centrifuged for 5 minutes at 8000g. The resulting supernatants were used as the DNA template. Templates were stored for up to 1 month at 4^{o}C.

Table 2-2: Bacterial control strains used for the AmpC plasmid gene PCR assays, CTX-M PCR assays and for the comparison of *E. coli ampC* promoter regions.

Organism	Purpose
Citrobacter freundii	CIT PCR Assay Control
Hafnia alvei	ACC PCR Assay Control
Klebsiella pneumoniae	DHA PCR Assay Control
Escherichia coli	FOX PCR Assay Control
Escherichia coli	MOX PCR Assay Control
Enterobacter cloacae	EBC PCR Assay Control
Escherichia coli (NCTC 12241)	Reference strain for wild-type
Escherichia coli (NCTC 13441)	CTX-M (Group 1) PCR Assay Control

The amount of DNA present in the templates was established for a selection of isolates during the optimisation stage. A Qubit 2.0 fluorimeter was used to calculate the amount of DNA present in 2.0µl of the templates. A total of 10 isolates, selected at random, were tested in triplicate. The average quantity of DNA present was calculated from the triplicate results (data available in the appendices; Section 9.1).

2.3.2 SYBR Green Real-Time PCR

SYBR Green PCR methodology was used throughout this study. The assay parameters were based on those recommended by the master-mix manufacturer (Qiagen, Manchester, UK). All PCR reactions were completed on the Smart Cycler II Real-Time PCR instrument (Cepheid, Sunnyvale, US), using the Smart Cycler DX software (Version 3.0).

Fluorescence was read after each cycle, at the analyser settings for FAM dye (Channel 1). This equated to an excitation wavelength of 450-495nm and an emission wavelength of 510-527nm. The cycle threshold values (CT) were determined by the instrument software, as were the melting temperatures (TM) of the amplified products.

The Quantifast SYBR Green master-mix kit (Qiagen, Manchester, UK) was used due to the fast-start property of the Taq DNA polymerase. This meant that a short initial holding stage (5 minutes) was required and enabled easier handling and stability of

reagents at room temperature during preparation. The master-mix was aliquotted and kept at -18^{o}C to prevent deterioration from repeated freeze-thaw cycles (Qiagen Ltd, 2010).

2.3.3 PCR Protocols

The standard PCR protocol (**Table 2-3**) used initially for method development and for the simplex assays was based on that recommended in the master-mix kit insert (Qiagen Ltd, 2010). The melting curve stage was added to confirm the presence of a PCR product at the end of the amplification stage. The annealing and extension stages were combined into a single stage (60^{o}C for 30 seconds), as recommended in the master-mix instructions.

Table 2-3: PCR protocol used for the simplex real-time PCR assays, and for initial assay development.

Stage	Protocol
Initial hold stage	95^{o}C for 5 minutes
Denaturation stage	95^{o}C for 10 seconds (35 cycles)
Annealing / extension stage	60^{o}C for 30 seconds (35 cycles)
Melting curve stage	60^{o}C to 95^{o}C, increasing at 0.5^{o}C / sec

The melting curve was used to confirm the presence of a PCR product post-amplification. The peaks observed on the melting curve graph were analogous to those seen in conventional end-point PCR by gel electrophoresis (Giglio, Monis & Saint, 2003). The peaks generated by intended products could easily be differentiated from peaks caused by non-specific amplification, due to the differences in melting temperatures.

2.3.4 Optimisation of Primer Concentration

The primers used initially for detecting the *ampC*-carrying plasmids were those published by Perez-Perez and Hanson (2002). Primers were purchased from an external supplier (Invitrogen, Paisley, UK), and were re-constituted on receipt in Tris-EDTA pH8.0 buffer (Tris 10mM, EDTA 1mM) (A0386,0500, VWR, Lutterworth, UK) to a working concentration of 100µM. Stock vials were stored at -18^{o}C for up to

12 months. Working stock solutions (2.5µM) of each primer were made up in Tris-EDTA buffer, and stored at 4^{o}C for up to 3 months.

The initial optimisation of primer concentrations was done by testing each of the six primer pairs at five different concentrations. The amount of primer solution added to each reaction tube was varied, so that primers were tested at 0, 0.08, 0.15, 0.25, 0.35 and 0.45µM final concentrations (**Table 2-4**).

Table 2-4: Reaction mixtures for primer optimisation, with each of the six AmpC plasmid primers tested at six different concentrations.

	0µM	0.08µM	0.15µM	0.25µM	0.35µM	0.45µM
Master-mix	12.5µl	12.5µl	12.5µl	12.5µl	12.5µl	12.5µl
Primer - F	-	0.8µl	1.5µl	2.5µl	3.5µl	4.5µl
Primer - R	-	0.8µl	1.5µl	2.5µl	3.5µl	4.5µl
Water	12.5µl	10.9µl	9.5µl	7.5µl	5.5µl	3.5µl
Total	25.0µl	25.0µl	25.0µl	25.0µl	25.0µl	25.0µl

Each reaction mixture comprised primers, 12.5µl SYBR Green master-mix and water making up the final volume to 25µl. Water was used in the place of a DNA template, so that any amplification present would be non-specific in nature. The assay PCR protocol described in 2.3.3 was used. CT and TM values for each assay were recorded, and the presence of non-specific product amplification was noted from the dissociation curve.

2.3.5 Specificity of Primers for Detecting *ampC*-Carrying Plasmids

In order to confirm the absence of cross-reactivity between the six AmpC plasmid control strains, each strain was tested against each of the six primer sets. Reaction mixtures comprised 12.5µl SYBR Green master-mix, 2.0µl each primer (0.2µM), 2.0 DNA template, made up to 25µl with water. The PCR assay protocol described in Section 2.3.3 was used.

2.3.6 Validation of Amplification Product

To ensure the amplification of intended products, the PCR amplification products were referred for sequencing at an external laboratory (Eurofins Genomics,

Germany). Each control strain was tested against its respective primer pair, using the PCR protocol described in 2.3.3 and primers at 0.2µM concentration. PCR products were sent for sequencing at the external laboratory, using the forward amplification primer as the sequencing primer. The reported sequence of each product was compared against the GenBank entry for each control strain (**Table 2-5**). Sequences were aligned using ClustalW2 software (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and the degree of sequence identity between sequences was determined by calculating the number of non-consensus nucleotides.

Table 2-5: The GenBank sequence entries used for the comparison of target product sequences for each of the bacterial control strains.

Control Strain	Gene / Enzyme	GenBank Acc No.
C. freundii (CIT)	LAT-1	X78117
H. alvei (ACC)	ACC-1	AJ133121
K. pneumoniae (DHA)	DHA-1	Y16410
E. coli (FOX)	FOX-1	X77455
E. coli (MOX)	MOX-1	D13304
Ent. cloacae (EBC)	MIR-1	M37839
E. coli (NCTC 12241)	*ampC*	AY899338
E. coli (NCTC 13441)	CTX-M-15	AY463958

2.4 Detection of AmpC Plasmid Genes

2.4.1 Review of Primers

Since the original AmpC plasmid primer sequences were published in 2002, the number of registered AmpC plasmid-mediated enzymes had increased significantly. Current plasmid-mediated enzymes are listed on a central, curated, website (www.lahey.org/studies/). At the time of checking (November 2011), there were 119 recorded AmpC plasmid-mediated enzymes, of which 94 had a registered GenBank sequence. Since November 2011, the number of registered plasmid-mediated enzymes has increased further, to 239 (last accessed 2nd February 2015). A full list of the current AmpC plasmid-mediated enzymes is provided in the appendices (Section 9.2).

The 94 AmpC plasmid gene sequences were downloaded from the GenBank website and aligned using the ClustalW2 software. Aligned sequences were checked against the original primers, to determine whether the more recently registered plasmid genes would continue to be detected. Potential new primers were identified from the consensus regions in the aligned sequences, and were checked using the Primer-BLAST software (www.ncbi.nlm.nih.gov/tools/primer-blast/) for specificity, product size, GC content and melting temperatures (Ye *et al.*, 2012).

The new primers (shown in **Table 2-6**) were validated by testing against the respective control strains using simplex PCR assays, using the same protocols as described in Section 2.3.6. Products were referred to the external laboratory for sequencing (Eurofins Genomics, Germany) to confirm the correct target had been amplified. Sequences were compared against the reference sequences for each of the AmpC plasmid gene groups (**Table 2-5**) using ClustalW2.

Table 2-6: Primers used for the two multiplex PCR assays for the detection of AmpC plasmid genes.

Primers	Sequences (5' to 3')	Product Size (bp)	Source
CIT2-F	TGA TGC AGG AGC AGG CTA TTC	417	++
CIT2-R	ACA GAC CAA TGC TGG AGT TAG		
ACC-F	AAC AGC CTC AGC AGC CGG TTA	346	**
ACC-R	TTC GCC GCA ATC ATC CCT AGC		
DHA-F	AAC TTT CAC AGG TGT GCT GGG T	405	**
DHA-R	CCG TAC GCA TAC TGG CTT TGC		
FOX-F	AAC ATG GGG TAT CAG GGA GAT G	175	Forward ++ Reverse **
FOX2-R	GAT TGG CCT GGA AGC TCA CCG		
MOX-F	GCT GCT CAA GGA GCA CAG GAT	322	Forward ++ Reverse **
MOX2-R	CAC CTC CTC GGG GAA TTG CAG		
EBC2-F	AAC TGG CAG CCG CAG TGG AAG	572	++
EBC2-R	GCC ACG TAG CTG CCA AAC CC		

++ This study

** Perez-Perez & Hanson (2002)

2.4.2 Multiplex Assay for Detecting AmpC Plasmid Genes

Using the updated primers, multiplex assays were developed from a combined primer solution, made up with 5µl of each primer (100µM) added to 100µl of Tris-EDTA buffer. A 4.0µl volume of the pooled primer solution was used in the assay,

together with 12.5µl master-mix, 2.0µl template and 6.5µl water. Primer combinations used were for CIT/ACC/DHA and FOX/MOX/EBC plasmid gene groups.

Assays were run for each of the relevant control strains. Initially, the same PCR protocol was used as for the simplex assays. However, a protocol with a longer extension / annealing time (**Table 2-7**) was subsequently found to give more consistent results. Each primer solution was tested against all of the relevant control strains.

Table 2-7: PCR assay protocol used for the AmpC plasmid gene multiplex assays

Stage	Protocol
Initial hold stage	95^{o}C for 5 minutes
Denaturation stage	95^{o}C for 10 seconds (35 cycles)
Annealing / extension stage	60^{o}C for 60 seconds (35 cycles)
Melting curve stage	60^{o}C to 95^{o}C, increasing at 0.5^{o}C / sec

The presence of AmpC plasmid genes in clinical isolates was determined using the two multiplex real-time SYBR Green assays as an initial screen. The screening assays were designed to detect groups of plasmid genes: CIT/DHA/ACC and FOX/MOX/EBC. Primers for the plasmid gene groups were as shown in **Table 2-6**. Multiplex assays with positive dissociation curves were confirmed using the same primers in three separate simplex reactions, to identify the individual AmpC plasmid gene present.

2.5 Sequencing the *E. coli ampC* Promoter Region

A 271bp region of the *ampC* gene, including the promoter region, attenuator region and part of the coding region was amplified for each isolate, using previously published primers (Caroff *et al.*, 2000) (**Table 2-8**).

Table 2-8: Primers used for the amplification of the *E. coli ampC* promoter region.

Primers	Sequences (5' to 3')	Product Size (bp)	Source
AMPC-F	GAT CGT TCT GCC GCT GTG	271	Caroff *et al*. (2000)
AMPC-R	GGG CAG CAA ATG TGG AGC AA		

Primers were used at a 0.1µM concentration. The simplex PCR protocol described in Section 2.3.3 was used. A melting curve protocol was run to confirm the presence of an amplified product prior to sequencing. PCR products were sent to an external company (Eurofins Genomics, Germany) for purification and sequencing. The forward amplification primer was used for sequencing.

The reported sequence of each product was compared against the GenBank entry for *E. coli* NCTC 12241 (GenBank Accession No AY899338), a laboratory control strain. Sequences were aligned using the ClustalW alignment tool in the Geneious software package (Version 7.1.7) (Biomatters, New Zealand). The sequence visualisation tool in Geneious was used to identify the nature and position of each mutation, with each polymorphism recorded according to the numbering system of Jaurin *et al*. (1981).

2.6 Detection of ESBL Resistance Genes

The presence of $bla_{CTX\text{-}M}$ genes was determined by using two simplex assays for CTX(M) Group 1 specific primers and universal CTX-M primers. The primers used for the assays are shown in **Table 2-9**.

Table 2-9: Primers used for the detection of CTX-M ESBL resistance genes.

CTX-M(P) indicates the universal primers and CTXM(1) indicates the Group 1-specific primers.

Primers	Sequences (5' to 3')	Product Size (bp)	Source
CTX-M(P)-F	TTT GCG ATG TGC AGT ACC AGT AA	544	Edelstein (2003)
CTX-M(P)-R	CGA TAT CGT TGG TGG TGC CAT A		
CTX-M(1)-F	AAA AAT CAC TGC GCC AGT TC	415	Woodford (2010)
CTX-M(1)-R	AGC TTA TTC ATC GCC ACG TT		

The Group 1 CTX-M specific primers were used as the initial screening assay. Isolates with a negative CTX-M Group 1 assay were also then tested for the presence of other CTX-M genes using the universal CTX-M primers (Edelstein *et al.*, 2003).

2.7 Multi-Locus Sequence Typing

The method developed by M. Achtman and others was used to determine the sequence type of each isolate (Wirth *et al.*, 2006). Fragments of seven housekeeping genes were amplified and sequenced (**Table 2-10**).

Table 2-10: The seven housekeeping genes used in the MLST method for *E. coli.*
(Wirth *et al.*, 2006)

Locus	Gene
adk	adenylate kinase
fumC	fumarate hydratase
gyrB	DNA gyrase
icd	isocitrate/isopropylmalate dehydrogenase
mdh	malate dehydrogenase
purA	adenylosuccinate dehydrogenase
recA	ATP/GTP binding motif

DNA was extracted from each isolate using the mechanical lysis method described in Section 2.3.1. Each gene was amplified using the primers shown in **Table 2-11**. Amplification was carried out in 25µl volumes; with 12.5µl master-mix, 3.0µl of each primer (2.5µM), 2.0µl of DNA template and 4.5µl of water. The primers used were those published on the central website, available at http://mlst.warwick.ac.uk/mlst/dbs/Ecoli/documents/primersColi.

The PCR protocol was adapted from that used by Wirth *et al*. (2006) and is shown in **Table 2-12**. Due to the larger sizes of the amplicons, and the need to have longer extension times and different temperatures, the protocol differs to those used in earlier sections. A melting curve protocol was run to confirm the presence of an amplified product prior to sequencing.

Table 2-11: Primers used to amplify the genes at each of the seven loci used for the *E. coli* MLST scheme.
(Wirth *et al.*, 2006)

Primer	Sequences (5' to 3')	Product Size (bp)
ADK2-F	TCA TCA TCT GCA CTT TCC GC	583
ADK2-R	CCA GAT CAG CGC GAA CTT CA	
FUMC-F	TCA CAG GTC GCC AGC GCT TC	806
FUMC-R	GTA CGC AGC GAA AAA GAT TC	
GYRB-F	TCG GCG ACA CGG ATG ACG GC	911
GYRB-R	ATC AGG CCT TCA CGC GCA TC	
ICD-F	ATG GAA AGT AAA GTA GTT GTT CCG GCA CA	878
ICD-R	GGA CGC AGC AGG ATC TGT T	
MDH-F	ATG AAA GTC GCA GTC CTC GGC GCT GCT GGC GG	932
MDH-R	TTA ACG AAC TCC TGC CCC AGA GCG ATA TCT TTC TT	
PURA-F	CGC GCT GAT GAA AGA GAT GA	816
PURA-R	CAT ACG GTA AGC CAC GCA GA	
RECA-F	CGC ATT CGC TTT ACC CTG ACC	780
RECA-R	TCT CGA TCA GCT TCT CTT TT	

Table 2-12: PCR protocol for the amplification of the seven housekeeping genes in the *E. coli* MLST scheme.

Stage	Protocol
Initial hold stage	95^{o}C for 5 minutes
Denaturation stage	95^{o}C for 30 seconds (30 cycles)
Annealing stage	56^{o}C for 30 seconds (30 cycles)
Extension stage	72^{o}C for 30 seconds (30 cycles)
Melting curve stage	60^{o}C to 95^{o}C, increasing at 0.5^{o}C / sec

PCR products were sent to an external company (Eurofins Genomics, Germany) for purification and sequencing. The forward amplification primer was used first for sequencing. The reported sequence of each product was entered onto an online database (Jolley & Maiden, 2010) (at http://pubmlst.org/) to obtain the allele profile. A profile number was accepted only if the database result indicated an exact match to the designated sequence. Once a full set of seven alleles had been identified, the sequence type was obtained from entering the 7-digit number into the same database. If a valid allele profile was not obtained for a locus, the process would be repeated again using the forward amplification primer and then (if still required) using the reverse amplification primer. Potential new alleles were

sequenced at least twice, and the raw data files submitted to the database curator for inclusion in the database and the generation of a new allele number. Isolates with a new sequence type were registered on the database website, so a new sequence type profile number could be generated.

2.8 Data Analysis

This study made use of the PubMLST website (available at http://.pubmlst.org/), developed by Keith Jolley and sited at the University of Oxford. The development of the website was funded by the Wellcome Trust.

Patient demographics and sample data were held in a MS Access database and analysed using MS Excel spreadsheets. The significance of differences between categorical variables was calculated using either Chi-square tests or Fisher's exact test. The online statistics software GraphPad QuickCalcs (available at www.graphpad.com/quickcalcs/) was used to calculate P values. A P value of <0.05 was considered to be an indicator of statistical significance.

The web-based ClustalW2 software (www.ebi.ac.uk/Tools/msa/clustalw2/) was used to compare the sequences of amplified products with known sequences downloaded from GenBank. All sequences were imported into the Geneious software package (Version 7.1.7). The package includes an alignment and visualisation tool for DNA sequences. The package also includes a plugin tool based on the ClustalW2 algorithm.

The web-based eBURST software (V3) was used to cluster the MLST sequence types into related groups (Feil *et al.*, 2004). The software is available at http://eburst.mlst.net/v3/. The eBURST software analyses the relationship between isolates grouped as clonal complexes, assigning sequence types as potential founders for a particular complex (Feil *et al.*, 2004). Clonal complexes were defined as a population of isolates with MLST alleles identical at six or more loci.

The Simpson's Index of Diversity (Hunter & Gaston, 1988) was used to determine the level of diversity between different bacterial populations. The 95% confidence

intervals for diversity indices were calculated using the method described by Grundmann *et al*. (2001).

2.9 Clinical Laboratories

A total of eight laboratories in the South West region were approached to participate in the study: Dorchester, Gloucester, Swindon, North Bristol, Bristol PHE, Torbay, Plymouth and Truro. These laboratories were selected to ensure a good geographic coverage of the region, and were of a size to ensure the number of isolates collected would meet the study requirements.

Figure 2-1: Locations of the five participating laboratories in the South West region: Gloucester, Swindon, Taunton, Dorchester and Truro.

Participating laboratories are indicated by a large black circle. Other laboratories in the region are indicated by a small yellow circle.
Map image used under the fair-use policy of Google Maps ©2012

Five of the laboratories were able to participate in the study (**Figure 2-1**). Local Trust approval was obtained for each laboratory via their Research & Development teams. The five final laboratories were Dorchester, Gloucester, Swindon, Taunton and Truro. Each laboratory was provided with an information sheet explaining the

purpose of the study and the instructions for submitting isolates (included in the appendices; Section 9.3).

2.10 Collection of Clinical Isolates

Clinical isolates of *E. coli* were collected from the five laboratories. Isolates for the pilot study (Gloucester only) were collected during the period June and November 2011. Isolates for the larger regional study were collected during the period April and May 2013.

Isolates were eligible for inclusion in the regional study provided the routine disc susceptibility testing indicated resistance to cefpodoxime (Public Health England, 2012). Laboratories were asked to submit only *E. coli* isolates for the study, but could submit isolates from any sample type. Laboratories were asked to exclude isolates if the same species had previously been isolated from the same patient within a 28-day period. This was to exclude the introduction of sampling bias from testing identical isolates from the same patient. Laboratories were asked to submit the first 50 isolates during the collection period; with the exception of the Gloucester laboratory, which collected 100 isolates.

Eligible isolates were allocated a unique study number by the local laboratory staff, with a prefix used to identify the laboratory of origin (DO- for Dorchester, G- for Gloucester, SW- for Swindon, TA- for Taunton and TR- for Truro). Laboratories were asked to submit the isolates on a suitable medium (usually a 3ml nutrient agar slope) and to provide basic demographic data for each isolate. A copy of the isolate information sheet for data collection is included in the appendices (Section 9.4). The information provided for each isolate included: specimen type, date of isolation, patient birth year, patient gender and whether the patient was an inpatient or outpatient / GP at the time the sample was taken. All isolates were anonymised at the point of collection, with the isolates identified subsequently only using the study reference number.

On receipt in the testing laboratory (Gloucester), isolates were sub-cultured onto a chromogenic urine media plate (Becton Dickinson, UK), to confirm the identity of

the isolate and the purity of the culture. This medium had previously been reported as having 99.3% specificity for *E. coli* based on the colour of the colonies (Merlino *et al.*, 1996). If required, further identification to species level was done using API20E identification strips (Biomerieux, UK). All isolates were then stored on cryobeads (Pro-Lab, UK) at -80^{o}C until required.

Isolates were excluded from the study if they were found not to be cefpodoxime-resistant or did not confirm as being *E. coli* when tested at the central laboratory. In the case of mixed cultures, a selection (up to three) of *E. coli* colonies was tested for cefpodoxime resistance. If present, a single resistant isolate was then included in the study.

2.11 Susceptibility Testing

Isolates were tested for a range of cephalosporins using a standardised disc susceptibility method, including the antibiotics cefpodoxime, cefuroxime, cefoxitin, cefotaxime and cefepime. Testing was performed according to the British Society of Antimicrobial Chemotherapy (BSAC) published guidelines (Andrews & Howe, 2011). To achieve a semi-confluent growth, a 0.5 MacFarland suspension was made and diluted to a 1:100 concentration. The final suspension was inoculated onto an Isosensitest agar plate (Oxoid) using a cotton-tipped swab. Antibiotic discs (Oxoid) were applied to the surface of the agar and the plate was incubated for 18-24 hours at 37^{o}C in air. Following incubation, the zone size for each antibiotic disc was recorded. The disc concentrations and respective zone sizes for result interpretations are shown in **Table 2-13**. Isolates were also tested for the presence of phenotypic ESBL activity using clavulanic acid synergy discs, with cefpodoxime as the indicator antibiotic (M'Zali *et al.*, 2000).

Gradient antibiotic strips (Oxoid) were used to test the cefotaxime minimum inhibitory concentration (MIC) within the range 0.002-32mg/L. A 0.5 MacFarland suspension was made and inoculated directly onto an Isosensitest agar plate (Oxoid) using a cotton-tipped swab. The gradient strip was placed on the surface of the agar and the plate was incubated for 18-24 hours at 37^{o}C in air. Following

incubation, the point of intersection of the zone to the strip was recorded as the MIC for the isolate.

Table 2-13: Antibiotic disc concentrations and recommended interpretive zone sizes (BSAC guidelines) for the five cephalosporins tested.
(Andrews & Howe, 2011)

Antibiotic	Disc Content (µg)	Resistant (mm)	Intermediate (mm)	Sensitive (mm)
Cefpodoxime	10	≤19	-	≥20
Cefuroxime	30	≤19	-	≥20
Cefotaxime	30	≤23	24-29	≥30
Cefoxitin	30	-	-	≥23
Cefepime	30	≤26	27-31	≥32

3 Development of PCR and Sequencing Methods

3.1 Introduction

This first stage of the study started in 2010, and was to develop and validate a suitable method for detecting beta-lactamase genes by real-time PCR. As a working clinical laboratory, the Gloucester laboratory had real-time PCR instruments already installed for diagnostic purposes. As this is the case for most clinical laboratories, and potential future utility was a consideration, it was decided that real-time SYBR Green PCR would be the primary DNA amplification and detection method. Offering a good alternative to traditional end-point PCR methods, key benefits of SYBR Green PCR chemistry include speed of assay times, ease of use and safer handling of reagents (Tong & Giffard, 2012).

Although real-time PCR can be used most effectively with fluorescent-labelled probes, Giglio *et al*. (2003) suggest that SYBR Green PCR chemistry is often used due to its relative inexpensive cost when compared to other detection methods. First used for monitoring PCR reactions in 1997 (Wittwer *et al.*, 1997), SYBR Green is an intercalating fluorescent dye that binds preferentially to double-stranded DNA. The use of melting curve analysis to visualise PCR products after the amplification stage gives melting curves that are analogous to the bands seen using gel electrophoresis (Giglio, Monis & Saint, 2003).

The original work on detecting the six AmpC plasmid gene groups used conventional PCR methods and gel electrophoresis (Perez-Perez & Hanson, 2002). Since then, Brolund *et al*. (2010) have demonstrated that real-time PCR using a SYBR Green dye and melting curve analysis can be used effectively to detect and classify the six AmpC plasmid gene groups in two multiplex assays.

The aim of this stage of the study was to develop a specific assay for the detection of bla_{AmpC} and $bla_{\text{CTX-M}}$ genes. A further aim was to develop the under-pinning methods and assays for the general amplification of PCR products prior to sequencing.

3.2 Methods

SYBR Green PCR methodology was used to develop a number of assays for the detection of key resistance genes in *E. coli*, and also for amplifying target genes prior to sequencing. All assays were run using the Smart Cycler II real-time PCR instrument (Cepheid, Sunnyvale, US), and the Quantifast SYBR Green master-mix (Qiagen, Manchester, UK).

A number of bacterial control strains were obtained, to act as PCR controls for the assays. Six strains (various species) were purchased from the national reference laboratory (Colindale, UK) for the six AmpC plasmid gene groups. Two further strains of *E. coli* were purchased from the National Collection of Type Cultures (NCTC); one to act as a positive control for the CTX-M PCR assays, and one to act as a reference wild type strain. Details of all control strains used are given in **Table 2-2**.

A crude lysis method for extracting bacterial DNA from isolates of *E. coli* was used for all assays (Woodford, 2010). The detection of the six AmpC plasmid gene groups was done initially using the primers published by Perez-Perez and Hanson (Perez-Perez & Hanson, 2002). These primers were later reviewed against the current list of AmpC plasmid gene sequences available in GenBank. The detection of CTX-M ESBL genes was done using a previously published universal set of CTX-M primers (Edelstein *et al.*, 2003) and also a set of Group 1 CTX-M specific primers (Woodford, 2010).

The amplification and sequencing of the *ampC* promoter region was done using the primers previously published by Caroff *et al.* (2000). These primers amplified a 271bp region of the *E. coli ampC* gene, including the promoter region, attenuator region and part of the coding region. Amplified PCR products were sent to an external company (Eurofins Genomics, Germany) for sequencing, using the forward amplification primer.

3.3 Results

3.3.1 Optimisation of DNA Template

The crude lysis method described by Woodford (2010) indicated that 2µl of the resulting DNA template would be sufficient for use with PCR assays. However, as this was originally based on using traditional end-point PCR, the optimum concentration level was established for the AmpC plasmid gene assays using real-time PCR. DNA quantification using the Qubit fluorimeter indicated an average amount of 2.4ng/µL in the lysate, with a range of 0.74 to 8.38ng/µL (see Appendices – Section 9.1). Although the DNA amount was not adjusted for each run, the same lysate was used within control strains.

Each control strain was tested against its respective primer pair, with the DNA template volume varied between 0µl and 4.0µl. The master-mix and primer concentrations (0.2µM) were kept consistent for all experiments, but the water was varied to make the final volume up to 25µl for each reaction. The CT values for the six control strains amplified with different template amounts are shown in **Table 3-1**.

Table 3-1: Cycle threshold (CT) values for the six AmpC plasmid gene assays; testing each bacterial AmpC plasmid control strain at six different DNA template amounts.

Primers used were those corresponding with each relevant bacterial control strain.

	DNA Template Volume					
Controls	**0µl**	**0.5µl**	**1.0µl**	**2.0µl**	**3.0µl**	**4.0µl**
CIT	-	29.6	27.8	26.9	26.7	25.9
ACC	-	25.5	24.2	22.5	22.2	20.9
DHA	-	26.7	25.5	24.9	23.9	23.1
FOX	-	23.7	20.2	19.4	18.4	18.2
MOX	-	33.7	26.8	26.0	23.5	22.8
EBC	-	22.5	20.9	19.5	18.8	18.1

The data demonstrate that all assays had a CT value of <30 when tested at the proposed template volume of 2.0µl. The melting temperatures for the products were consistent within each strain (**Table 3-2**), with <0.5^{o}C variation from the mean value for each control strain.

Table 3-2: Melting temperature (TM) values for the products of the bacterial AmpC plasmid control strains when tested at six different template amounts.

The value for CIT at 1.0µl (indicated in green) did not register a specific product peak at >80^{o}C. This value was not included in the average TM value calculation. Ave = average.

	DNA Template Volume					
Controls	**0.5µl**	**1.0µl**	**2.0µl**	**3.0µl**	**4.0µl**	**Ave**
CIT	87.9	68.8	87.9	88.0	87.6	87.6
ACC	87.0	86.9	86.7	87.1	86.9	86.9
DHA	89.3	89.3	89.2	89.4	89.4	89.3
FOX	89.0	88.9	89.2	89.3	89.2	89.1
MOX	91.4	91.0	91.0	90.9	90.7	91.0
EBC	89.8	89.3	89.3	89.6	89.9	89.6

3.3.2 Optimisation of Primer Concentrations for AmpC Plasmid Gene PCR

A series of experiments was undertaken to determine the optimum initial concentration for the primers in the AmpC plasmid gene PCR assays. Primers for each of the six AmpC plasmid gene groups were tested at five different concentrations, using water as a blank template. The purpose of the experiments was to assess the level of background amplification caused by the non-specific binding nature of SYBR Green (Ririe, Rasmussen & Wittwer, 1997). The concentrations of primers ranged from 0.08µM to 0.45µM (**Table 3-3**).

Table 3-3: Cycle threshold (CT) values indicating non-specific amplification in the six AmpC plasmid gene assays.

Cycle threshold values are given for the six AmpC plasmid gene PCR assays at different primer concentrations. Assays were run with water as the template.

Primers	**0.08µM**	**0.15µM**	**0.25µM**	**0.35µM**	**0.45µM**
CIT	0	0	32.1	33.5	31.3
ACC	0	0	0	33.3	0
DHA	0	0	32.1	33.8	27.9
FOX	0	0	0	0	0
MOX	0	0	0	28.1	33.1
EBC	0	33.2	31.4	29.1	30.3

The purpose of optimising a single concentration for all primers was to develop a single protocol base for all assays, in which only the primers would differ. This would simplify the protocol for future clinical utility. Overall, the amount of non-

specific amplification increased with the rising concentration of primers. None of the primers demonstrated non-specific amplification at the 0.08µM concentration, and this was also the case for the majority of primers at the 0.15µM concentration. The FOX primers were the only primers not to exhibit non-specific amplification throughout the concentration range. A concentration of 0.2µM was chosen as the mid-point between the two tested concentrations of 0.15µM and 0.25µM.

The presence of a non-specific product could clearly be identified through the use of the dissociation graph (**Figure 3-1**). The range of melting temperatures observed for all primer groups was 65.8°C to 81.9°C for the non-specific amplification, with an average temperature of 79.2°C. The height of the melting curve peak was higher in the assays with a greater primer concentration.

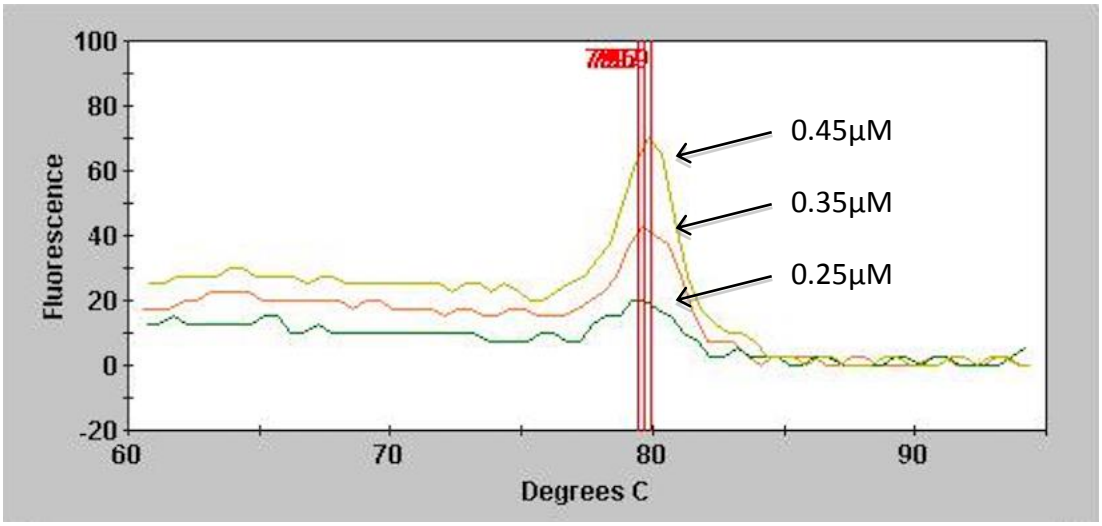


Figure 3-1: Evidence of non-specific amplification in a CIT plasmid gene real-time PCR assay using SYBR Green chemistry.

Primers were tested at five different concentrations (0.08µM to 0.45µM), with water used as the template. The melting curves for 0.25, 0.35 and 0.45µM are shown.

3.3.3 Validation of AmpC Control Strains

Having selected an initial primer concentration of 0.2µM from the experiments in Section 3.3.2, the set of six bacterial control strains for the AmpC plasmid gene groups was checked to confirm specificity for each of the relevant primer sets. Each control strain was tested against each of the six primer sets. This also confirmed

that the primer concentration of 0.2µM enabled detection of all control strains at that level.

The CT values for the primers tested against each control strain are shown in **Table 3-4**. With the exception of the CIT primers, each assay produced a CT value of <30 when tested against the respective control strain. The presence of a non-specific amplification product was noted in 9 of the 30 expected "negative" assays, although the average CT values were significantly higher (31.6 versus 26.7, p=0.007, χ^2 test). The assay for the FOX control strain tested with the MOX primers did give a CT value of 27.2. However, the melting temperature of this product was 69.1^{o}C and was due to non-specific amplification.

Table 3-4: Cycle threshold (CT) values for the six AmpC plasmid gene assays; testing each control strain against all of the six primer sets.

The values shaded in green are those were a true positive result was expected. The presence of CT values in the other assays was the result of non-specific amplification.

	Bacterial Control Strains (CT values)					
Primers	**CIT**	**ACC**	**DHA**	**FOX**	**MOX**	**EBC**
CIT	31.3	0	0	32.3	0	30
ACC	0	24.7	0	32.8	0	32.9
DHA	0	0	26.5	0	0	30.2
FOX	0	0	0	28.4	0	33.9
MOX	0	0	0	27.2	28.9	31.2
EBC	34.1	0	0	0	0	20.5

The presence of a non-specific product could be clearly differentiated from the target product using the dissociation curve (**Figure 3-2**). Not only is there a clear, and higher peak for the true positive peak, but there is also a significant shift to the left for the non-specific products. Non-specific amplification often results in smaller products with lower melting temperatures (Ririe, Rasmussen & Wittwer, 1997).

All melting temperatures (TM) for the true positive products were >85^{o}C, with a range of 86.8^{o}C to 91.3^{o}C (**Table 3-5**). Non-specific amplification was observed in some tubes, but within the range 65.4 to 81.5^{o}C.

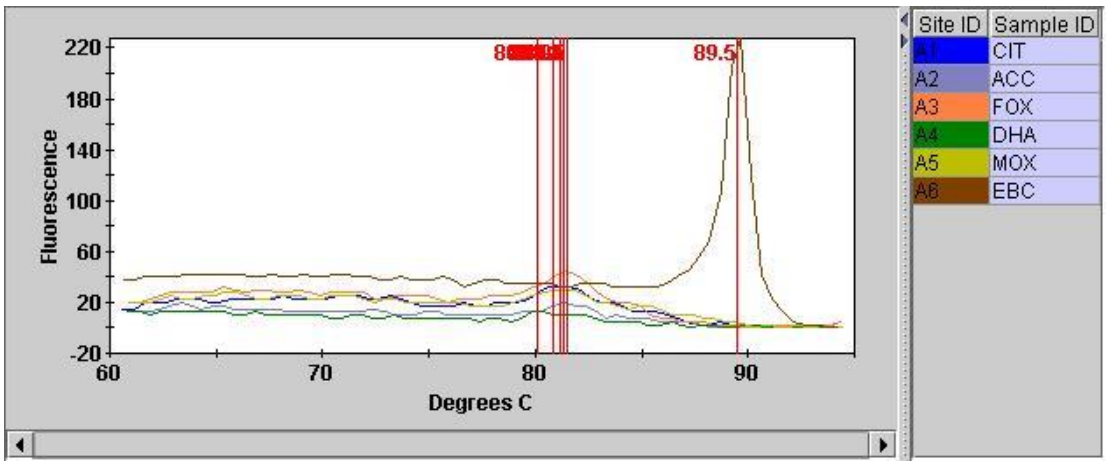


Figure 3-2: Melting curve graphs of an EBC plasmid gene assay testing each of the six AmpC bacterial control strains with the EBC primers.

Some evidence is present of low-level non-specific amplification at a TM in the region of 81°C compared to the target EBC plasmid gene product at 89.5°C.

Table 3-5: Melting temperature (TM) values for the amplified products of control strains when tested against all six of the AmpC plasmid gene primer sets.

The values shaded in green are those where a true positive result was expected. The presence of a TM value in the other assays indicates the presence of non-specific amplification.

	Bacterial Control Strains (TM)					
Primers	**CIT**	**ACC**	**DHA**	**FOX**	**MOX**	**EBC**
CIT	87.6	65.4	0	80.2	68.1	80.9
ACC	0	86.8	0	79.2	65.8	81.4
DHA	0	0	89.2	0	66	81.5
FOX	0	0	65.4	89.2	70.4	80.1
MOX	0	0	0	69.1	91.3	81.1
EBC	80.4	0	0	0	66.6	89.5

3.3.4 Validation of Amplification Product

To confirm that the AmpC plasmid gene assays were detecting and amplifying the correct product, the resulting PCR products were referred for sequencing by an external company (Eurofins Genomics, Germany). All products were sequenced using the respective forward amplification primer. Sequencing was performed using the dideoxy chain termination method on ABI3730XL instruments (Applied Biosystems). The resulting DNA sequences were compared against the relevant sequences in GenBank, using the online ClustalW software: (www.ebi.ac.uk/Tools/msa/ClustalW2/).

The sequences of all six AmpC plasmid gene products showed a good degree of sequence identity (>92%) when compared to the GenBank sequence (**Table 3-6**). An example of one of the ClustalW analysis reports is shown in **Figure 3-3**, with the other reports provided in the appendices (Section 9.5).

Table 3-6: Degree of sequence identity of each of the six sequenced AmpC plasmid gene products, when compared with the respective reference sequence.

GenBank accession numbers are given for each reference sequence.

Control Strain	GenBank Acc.	Total Bases	Consensus Bases	% Identity
CIT	X78117	356	335	94.1
ACC	AJ133121	306	302	98.7
DHA	Y16410	335	335	100.0
FOX	X77455	142	131	92.3
MOX	D13304	472	469	99.4
EBC	M37839	261	258	98.9

The process was repeated for the two sets of CTX-M primers used (universal and Group 1-specific). Both sets of primers were used to amplify the respective regions in the control strains *E. coli* NCTC 13441. The PCR products were referred for sequencing, using the forward amplification primers. The resulting sequences were aligned with the GenBank sequence for the CTX-M-15 gene (Accession No. AY463958) using the ClustalW online software. The alignment output for the universal CTX-M primers is shown in **Figure 3-4**. The ClustalW alignment report for the Group 1- specific primers is provided in the Appendices (Section 9.6). The degree of sequence identity for the universal and Group 1-specific primers was 99.6% and 98.2%, respectively.

```
CIT-1_CIT-F                -----------------------GTTACGGATAAAGCCGAATTACTGCGC 27
CIT-2_CIT-F                TGCCGCTGCAGATCCCCGATGACGTTACGGATAAAGCCGAATTACTGCGC 83
gi|496632|emb|X78117.1|    TACCGCTGCAGATCCCCGATGACGTTAGGGATAAAGCCGCATTACTGCAT 600
                                                 **** *********** ********

CIT-1_CIT-F                TTTTATCAAAACTGGCAACCACAATGGACTCCGGGCGCTAAGCGTCTTTA 77
CIT-2_CIT-F                TTTTATCAAAACTGGCAACCACAATGGACTCCGGGCGCTAAGCGTCTTTA 133
gi|496632|emb|X78117.1|    TTTTATCAAAACTGGCAGCCGCAATGGACTCCGGGCGCTAAGCGACTTTA 650
                           ***************** ** ********************** *****

CIT-1_CIT-F                CGCTAACTCCAGCATTGGTCTGTTTGGTGCGCTGGTGGTAAAACATTCAG 127
CIT-2_CIT-F                CGCTAACTCCAGCATTGGTCTGTTTGGTGCGCTGGTGGTAAAACATTCAG 183
gi|496632|emb|X78117.1|    CGCTAACTCCAGCATTGGTCTGTTTGGCGCGCTGGCGGTGAAACCTTCAG 700
                           *************************** ******* *** **** *****

CIT-1_CIT-F                GTATGAGCTACGAAGAGGCAATGACCAGACGCGTCCTGCAACCATTAAAA 177
CIT-2_CIT-F                GTATGAGCTACGAAGAGGCAATGACCAGACGCGTCCTGCAACCATTAAAA 233
gi|496632|emb|X78117.1|    GAATGAGTTACGAAGAGGCAATGACCAGACGCGTCCTGCAACCATTAAAA 750
                           * ***** ****************************************

CIT-1_CIT-F                CTGGCGCATACCTGGATTACGGTTCCGCAAAGCGAACAAAAAAATTATGC 227
CIT-2_CIT-F                CTGGCGCATACCTGGATTACGGTTCCGCAAAGCGAACAAAAAAATTATGC 283
gi|496632|emb|X78117.1|    CTGGCGCATACCTGGATTACGGTTCCGCAGAACGAACAAAAAGATTATGC 800
                           **************************** * ********** *******

CIT-1_CIT-F                CTGGGGCTATCGCGAAGGGAAGCCTGTACACGTTTCTCCGGGGCAACTTG 277
CIT-2_CIT-F                CTGGGGCTATCGCGAAGGGAAGCCTGTACACGTTTCTCCGGGGCAACTTG 333
gi|496632|emb|X78117.1|    CTGGGGCTATCGCGAAGGGAAGCCCGTACACGTTTCTCCGGGACGACTTG 850
                           ********************** ********** * *****

CIT-1_CIT-F                ACGCCGAAGCCTATGGCGTGAAATCCAGCGTTATCGATATGGCCCGCTGG 327
CIT-2_CIT-F                ACGCCGAAGCCTATGGCGTGAAATCCAGCGTTATCGATATGGCCCGCTGG 383
gi|496632|emb|X78117.1|    ACGCCGAAGCCTATGGCGTGAAATCCAGCGTTATTGATATGGCCCGCTGG 900
                           ********************************** ***************

CIT-1_CIT-F                GTTCAGGCCAACATGGACGCCAGCCACGTTCAGGAGAAA----------- 366
CIT-2_CIT-F                GTTCAGGCCAACATGGACGCCAGCCACGTCAAAG---------------- 417
gi|496632|emb|X78117.1|    GTTCAGGCCAACATGGATGCCAGCCACGTTCAGGAGAAAACGCTCCAGCA 950
                           ***************** ***********  * *
```

Figure 3-3: ClustalW alignment report for the sequenced products of the CIT control strain tested in duplicate using the CIT primers.

The GenBank sequence X78117 is included for comparison.
* indicates agreement at that position with all three nucleotides.

```
gi|39545937|gb|AY463958.1      GCAGCACCAGTAAAGTGATGGCCGCGGCCGCGGTGCTGAAGAAAAGTGAA 450
EC-13441_ctxmp-f               ----------------------------------------GAAGTGAA 8
                                                                       *******

gi|39545937|gb|AY463958.1      AGCGAACCGAATCTGTTAAATCAGCGAGTTGAGATCAAAAAATCTGACCT 500
EC-13441_ctxmp-f               -GCGAACCGAATCTGTTAAATCAGCGAGTTGAGATCAAAAAATCTGACCT 57
                                ************************************************

gi|39545937|gb|AY463958.1      TGTTAACTATAATCCGATTGCGGAAAAGCACGTCAATGGGACGATGTCAC 550
EC-13441_ctxmp-f               TGTTAACTATAATCCGATTGCGGAAAAGCACGTCAATGGGACGATGTCAC 107
                               ************************************************

gi|39545937|gb|AY463958.1      TGGCTGAGCTTAGCGCGGCCGCGCTACAGTACAGCGATAACGTGGCGATG 600
EC-13441_ctxmp-f               TGGCTGAGCTTAGCGCGGCCGCGCTACAGTACAGCGATAACGTGGCGATG 157
                               ************************************************

gi|39545937|gb|AY463958.1      AATAAGCTGATTGCTCACGTTGGCGGCCCGGCTAGCGTCACCGCGTTCGC 650
EC-13441_ctxmp-f               AATAAGCTGATTGCTCACGTTGGCGGCCCGGCTAGCGTCACCGCGTTCGC 207
                               ************************************************

gi|39545937|gb|AY463958.1      CCGACAGCTGGGAGACGAAACGTTCCGTCTCGACCGTACCGAGCCGACGT 700
EC-13441_ctxmp-f               CCGACAGCTGGGAGACGAAACGTTCCGTCTCGACCGTACCGAGCCGACGT 257
                               ************************************************

gi|39545937|gb|AY463958.1      TAAACACCGCCATTCCGGGCGATCCGCGTGATACCACTTCACCTCGGGCA 750
EC-13441_ctxmp-f               TAAACACCGCCATTCCGGGCGATCCGCGTGATACCACTTCACCTCGGGCA 307
                               ************************************************

gi|39545937|gb|AY463958.1      ATGGCGCAAACTCTGCGGAATCTGACGCTGGGTAAAGCATTGGGCGACAG 800
EC-13441_ctxmp-f               ATGGCGCAAACTCTGCGGAATCTGACGCTGGGTAAAGCATTGGGCGACAG 357
                               ************************************************

gi|39545937|gb|AY463958.1      CCAACGGGCGCAGCTGGTGACATGGATGAAAGGCAATACCACCGGTGCAG 850
EC-13441_ctxmp-f               CCAACGGGCGCAGCTGGTGACATGGATGAAAGGCAATACCACCGGTGCAG 407
                               ************************************************

gi|39545937|gb|AY463958.1      CGAGCATTCAGGCTGGACTGCCTGCTTCCTGGGTTGTGGGGGATAAAACC 900
EC-13441_ctxmp-f               CGAGCATTCAGGCTGGACTGCCTGCTTCCTGGGTTGTGGGGGATAAAACC 457
                               ************************************************

gi|39545937|gb|AY463958.1      GGCAGCGGTGGCTATGGCACCACCAA-CGATATCGCGGTGATCTGGCCAA 949
EC-13441_ctxmp-f               GGCAGCGGTGGCTATGGCACCACCAAACGATATC---------------- 491
                               ************************** *******
```

Figure 3-4: ClustalW alignment report for the sequenced product of the CTX-M control strain tested using the universal CTX-M primers.

The GenBank reference sequence (AY463958) for the CTX-M-15 gene was used for comparison.
* indicates agreement at that position for both nucleotides.

3.3.5 Review of AmpC Plasmid Gene Primer Sequences

When the original AmpC plasmid gene primers were published in 2002 (Perez-Perez & Hanson, 2002), there were 29 genes recorded and available for download from GenBank. At the time of this current review in November 2011, the number had increased to 119 recorded plasmid genes (94 available for download). Since then, the number has increased further still, with 239 AmpC plasmid genes currently recorded on the central website (www.lahey.org/studies/, last accessed 2nd February 2015). The full list of current plasmid genes is included in the appendices (Section 9.2). The list of 94 plasmid genes with available GenBank sequence entries at the time of the review is given in **Table 3-7**. The sequences for the 55 available CMY plasmid genes were aligned in ClustalW and checked for suitability against the original primers. It was clear that the original primers for the CIT group were no longer suitable, as the forward and reverse primers were not consensus with the sequence derived from the combined alignment (see Appendices, Section 9.7). The sequence for CMY-13 (GenBank Accession X92520) was not included in the ClustalW alignment analysis, as it had a large number of additional nucleotides throughout the sequence and therefore made full alignment difficult. It was possible from the remaining 54 CMY plasmid gene sequences to select new forward and reverse primers (**Table 3-8**), using new consensus regions in the alignment.

In contrast to the large number of additional CIT plasmid genes recorded since 2002, there had been only two plasmid genes added to the ACC group (ACC-3 and ACC-4). The ClustalW analysis of the four ACC sequences together indicated that no suitable consensus sequences were available for primer selection. The longest region of sequence agreement in the alignment was 6 base pairs in length, making the selection of suitable primers impossible. The ClustalW analysis for the review of the ACC plasmid genes is included in the appendices (Section 9.8).

Table 3-7: List of 94 plasmid *ampC* genes recorded on the www.lahey.org/studies/ website (Nov 2011) that were available for download from GenBank, together with relevant accession numbers.

Plasmid Gene	GenBank
CMY-1	X92508
CMY-2	X92509
CMY-3	X92510
CMY-4	X92511
CMY-5	X92512
CMY-6	X92513
CMY-7	X92514
CMY-8	X92515
CMY-9	X92516
CMY-10	X92517
CMY-11	X92518
CMY-12	X92519
CMY-13	X92520
CMY-14	X92521
CMY-15	X92522
CMY-16	X92523
CMY-17	X92524
CMY-18	X92525
CMY-19	X92526
CMY-20	X92527
CMY-21	X92528
CMY-22	X92529
CMY-23	X92530
CMY-24	X92531
CMY-25	X92532
CMY-26	X92533
CMY-27	X92534
CMY-28	X92535
CMY-29	X92536
CMY-30	X92537
CMY-31	X92538
CMY-32	X92539
CMY-33	X92540
CMY-34	EF394370
CMY-35	EF394371
CMY-36	EU331426
CMY-37	AB280919
CMY-38	AM931008
CMY-39	AB372224
CMY-40	EU515251
CMY-41	AB429270
CMY-43	FJ360626
CMY-44	FJ437066
CMY-45	FN546177
CMY-47	HM046998
CMY-48	HM569226
CMY-49	GQ402541

Plasmid Gene	GenBank
CMY-54	HM544039
CMY-55	HM544040
CMY-56	HQ322613
CMY-57	HQ285243
CMY-58	HQ185697
CMY-59	AB587082
CMY-64	HQ832678
CMY-73	GQ351345
ACC-1	AJ133121
ACC-2	AF180952
ACC-3	AF180958
ACC-4	EF504260
ACT-1	U58495
ACT-2	AM076977
ACT-3	EF125013
ACT-4	EU427302
ACT-5	FJ237369
ACT-6	FJ237366
ACT-7	FJ237368
CFE-1	AB107899
DHA-1	Y16410
DHA-2	AF259520
DHA-3	AY494945
DHA-5	JF273491
DHA-6	HQ322612
DHA-7	HQ456945
FOX-1	X77455
FOX-2	Y10282
FOX-3	Y11068
FOX-4	AJ277535
FOX-5	AY007369
FOX-6	AY034848
FOX-7	AJ703795
FOX-9	JF896803
LAT-1	X78117
MIR-1	M37839
MIR-2	AY227752
MIR-3	AY743435
MIR-4	EF417572
MIR-5	FJ237367
MOX-1	D13304
MOX-2	AJ276453
MOX-3	EU515248
MOX-4	FJ262599
MOX-5	GQ152600
MOX-6	GQ152601
MOX-7	GQ152602

Table 3-8: Updated primers for the CIT, FOX, MOX and EBC AmpC plasmid gene assays, following the review of aligned sequences for the six AmpC plasmid gene groups.

F indicates a forward primer, and R indicates a reverse primer.
(2) indicates an updated version of the primer.

Primers	Sequences (5' to 3')	Product Size (bp)
CIT(2)-F	TGATGCAGGAGCAGGCTATTC	417
CIT(2)-R	ACAGACCAATGCTGGAGTTAG	
FOX-F	As original primer	175
FOX(2)-R	GATTGGCCTGGAAGCTCACCG	
MOX-F	As original primer	322
MOX(2)-R	CACCTCCTCGGGGAATTGCAG	
EBC(2)-F	AACTGGCAGCCGCAGTGGAAG	572
EBC(2)-R	GCCACGTAGCTGCCAAACCC	

The original DHA primers were found to still have full sequence agreement with the six aligned DHA plasmid gene sequences. For the FOX and MOX plasmid gene groups, although the forward primers were found to still be suitable, the reverse primers were both non-consensus with the alignment of the newer plasmid genes. New reverse primers for both FOX and MOX were identified from their respective alignment sequences (**Table 3-8**)

As with the FOX and MOX primers, the review of the EBC primers showed that one primer was no longer suitable (forward primer in this case). An alternative primer pair was identified using the original reverse primer as a new forward primer. A new reverse primer was then identified from the remaining aligned sequence. The two new primers are shown in **Table 3-8**. The largest region of consensus sequences was 20bp in length, which restricted the selection of primers.

The four new primer pairs were tested against their respective control strains. All the primer pairs gave a good clear product peak on the dissociation curves. The corresponding CT and TM values are shown in **Table 3-9**. The melting temperatures were slightly different to those using the original primers. CIT, FOX, MOX and EBC originally had melting temperatures of 87.6^{o}C, 89.1^{o}C, 91.0^{o}C and 89.6^{o}C, respectively. All potential new primers were tested using the Primer-BLAST software (available at www.ncbi.nlm.nih.gov/tools/primer-blast/) to confirm the

accuracy of the primers, the melting temperatures of the products and the GC content of the primers and products.

Table 3-9: Cycle threshold (CT) and melting temperature (TM) values for the AmpC plasmid gene simplex assays using the updated primers for CIT, FOX, MOX and EBC plasmid gene groups.

Control	Primer Conc (µM)	CT	TM (°C)
CIT	0.2	23.7	88.9
FOX	0.2	20.3	88.0
MOX	0.2	19.5	90.4
EBC	0.2	26.0	89.3

As with the original primer sets, the PCR products from the assays with the revised primers were referred for sequencing. The ClustalW alignments of the resulting sequences showed a good degree of sequence identity with the respective GenBank sequences (**Table 3-10**). The level of sequence identity for the new CIT and FOX primers was reduced slightly when compared to the original primers (94.1% and 92.3%, respectively).

Table 3-10: Degree of pairwise identity (%) for the amplification products obtained with the new primer pairs for the CIT, FOX, MOX and EBC plasmid gene groups, when compared with the reference plasmid gene sequences.

GenBank accession numbers are given for each reference sequence.

Control Strain	GenBank Sequence	Total Bases	Consensus Bases	% Identity
CIT	X78117	370	346	93.5
FOX	X77455	124	112	90.3
MOX	D13304	284	282	99.3
EBC	M37839	522	518	99.2

3.3.6 Development of Multiplex Assays

In order to simplify the analysis of large numbers of isolates, multiplex assays were devised for the six AmpC plasmid gene groups. Two assays were designed, each containing three of the plasmid gene groups. One multiplex assay included the CIT, ACC and DHA plasmid gene groups and the other assay included the FOX, MOX and EBC groups. A positive assay could be seen on the melting curve analysis as a

distinct peak for each product (**Figure 3-5**). A similar approach was taken by Brolund *et al.* (2010), who also used SYBR Green real-time PCR. The CT and TM results of the CIT/ACC/DHA and FOX/MOX/EBC multiplex assays are shown in **Table 3-11**.

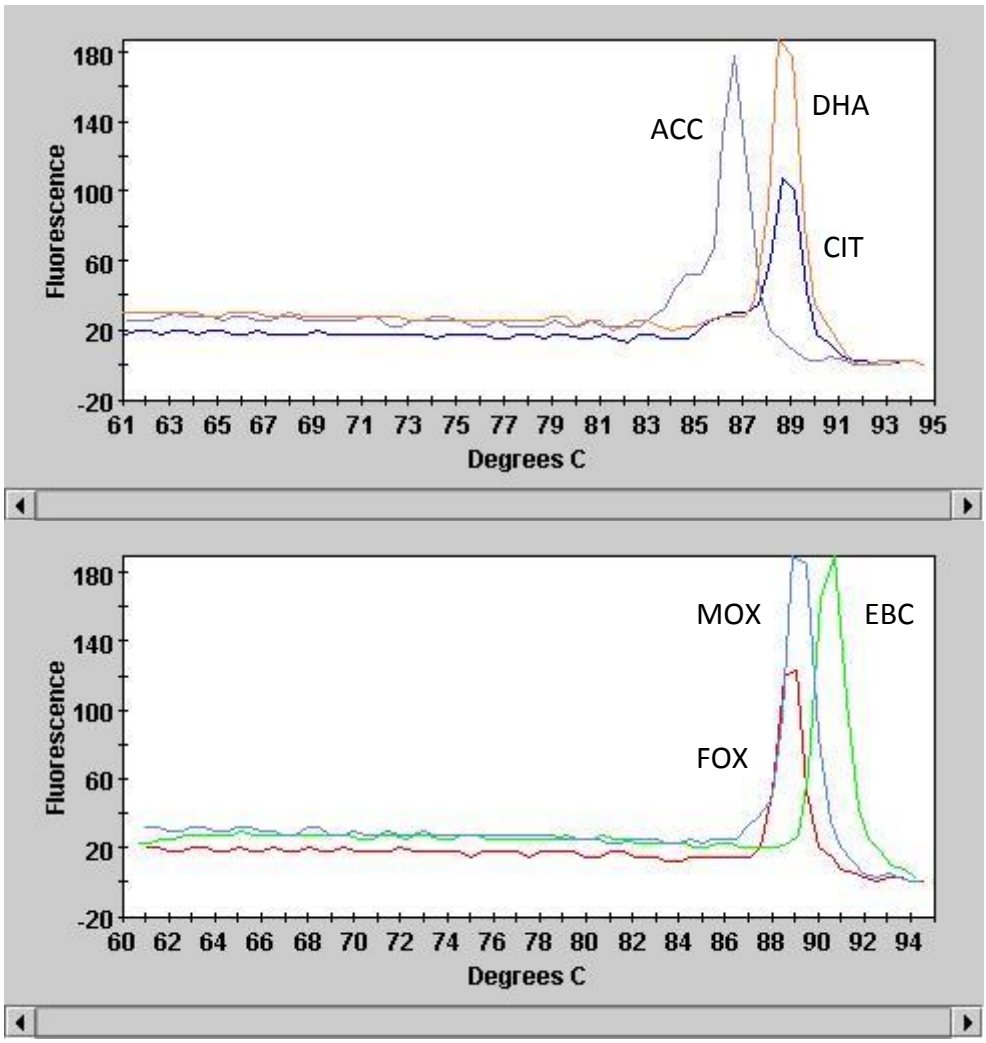


Figure 3-5: Melting curve graphs for the two AmpC plasmid gene multiplex assays.

Each of the AmpC bacterial control strains produced a characteristic peak at the expected melting temperature. The multiplex assays were split into two groups: A) for the CIT/ACC/DHA plasmid gene groups and B) for the FOX/MOX/EBC plasmid gene groups.

Table 3-11: Results of two multiplex assays for CIT, ACC, DHA and FOX, MOX, EBC using the combined primer solutions.

The negative assays (Neg) were run using water as a blank DNA template.

Assay (Combined Primers)	Control	CT Value	TM Value (^{o}C)
CIT/ACC/DHA	CIT	15.7	88.8
	ACC	13.6	86.6
	DHA	15.5	88.8
	Neg	27.0	80.8
FOX/MOX/EBC	FOX	17.5	88.8
	MOX	15.0	90.5
	EBC	13.2	89.2
	Neg	25.1	81.2

It was noted that the level of non-specific amplification appeared to be higher with the use of the pooled primers. In order to establish whether this was the case, a blank run of both the multiplex primer solutions were tested, using water in place of DNA template. The presence of non-specific amplification could be seen on the dissociation curve (**Figure 3-6**), with melting temperatures in the region of 81^{o}C. The level of non-specific amplification appeared to be lower in the presence of a "positive" amplification peak, due probably to competition for the master-mix components (Higuchi *et al.*, 1993).

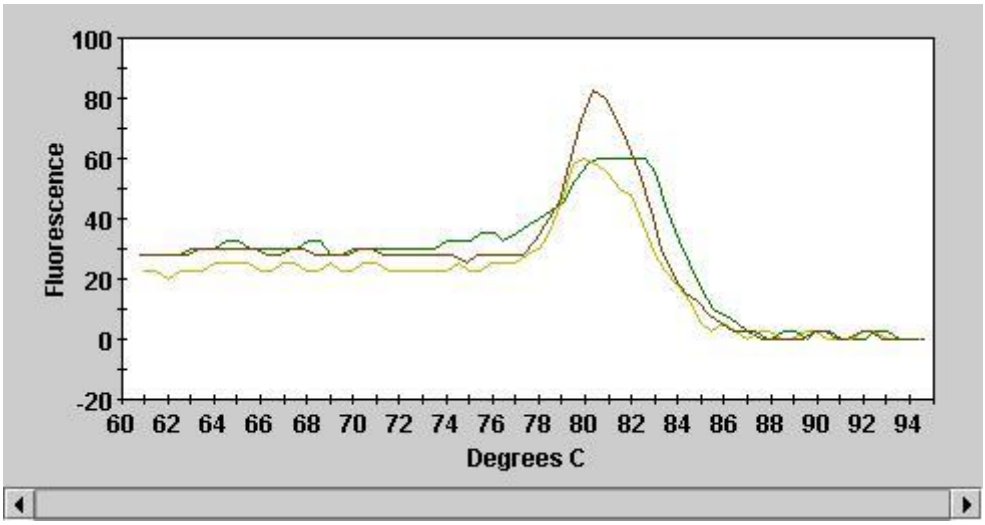


Figure 3-6: Melting curves for a series of three blank multiplex assays using the CIT/ACC/DHA pooled primers.

Evidence of non-specific amplification is present consistently at the 81^{o}C point in the three assays.

3.4 Discussion

3.4.1 Use of SYBR Green PCR and Melting Curve Analysis

The results from the method development stage demonstrated that a quick and accurate assay could be implemented using SYBR Green real-time PCR for the detection of acquired bla_{AmpC} genes. The assay protocol was easily amended to use different primers for different target DNA, such as $bla_{CTX\text{-}M}$ genes. The results here demonstrate robust assay performance for both the AmpC plasmid gene groups and the CTX-M ESBL genes, with a similar utility also for pre-sequencing amplification. The basic simplex assay protocol took approximately 43 minutes to complete, including the melting curve protocol. At the end of this period, the presence of the target gene could be easily determined from the melting curve analysis without the need for further handling.

The development stage included the important components of creating a new PCR assay. The optimal primer concentrations were determined using a set of previously published primers (Perez-Perez & Hanson, 2002). This enabled a useful initial benchmark to be set for future evaluation. Although none of the assays included an internal amplification control, assays were run with a positive and negative control sample included where relevant (Public Health England, 2013). In the case of the negative control, this was always PCR-grade water. In the case of the positive controls, it was one of the bacterial control strains used for this purpose. The AmpC plasmid control strains were obtained from the national reference laboratory and were reported to be those used in their AmpC plasmid gene PCR assay (Woodford, 2010 personal communication). Other control strains were obtained from the National Collection for Type Cultures (NCTC, Colindale). The specificity of each of the AmpC plasmid control strains was confirmed by the absence of cross-reactivity with other primers (**Table 3-4**). In the case of all the control strains, the PCR products were referred for sequencing to confirm that the correct target had been amplified by each assay. This also helped to confirm the suitability of the AmpC plasmid control strains as PCR controls, since they had not been purchased as recognised NCTC strains.

SYBR Green PCR technology and melting curve analysis have been reported to offer a robust solution for the detection of beta-lactamase genes. Brolund *et al*. (2010) have shown previously that the SYBR Green real-time PCR method could be used for the detection of AmpC plasmid-mediated genes, and can be used in the form of two triplex assays. Although some of the primers were updated in the Brolund paper to improve detection of the newer plasmid genes at the time, the assays were based largely on the original primers described by Perez-Perez and Hanson (2002). The decision to include the CIT, ACC and DHA primers together in the same multiplex in the current study was based on the prevalence reported in the UK for these plasmid-mediated enzymes (Woodford *et al.*, 2007). Although not tested on clinical isolates of other species of Enterobacteriaceae in the current study, some of the AmpC plasmid control strains included *Klebsiella pneumoniae*, *Citrobacter freundii* and *Enterobacter cloacae*. The simplex and multiplex assays were also shown to detect the presence of the AmpC plasmid genes in these strains as well as the *E. coli* strains. The multiplex assays developed in the current study, therefore, would have a similar utility in detecting bla_{AmpC} genes in these species isolated from clinical samples. The decision to group CIT, ACC and DHA together was taken so that a single PCR assay could be run as a screening assay to detect the most likely plasmid gene groups present in a wide range of clinical isolates. This would offer the important benefits to a diagnostic laboratory of increased speed and reduced cost.

One of the authors on the original plasmid AmpC PCR paper (Hanson) has subsequently published other work on the use of real-time PCR and fluorescent probe-labelled dyes for the detection of plasmid genes. In the paper by Geyer *et al.* (2012), they used fluorescent FAM-labelled probes to detect the presence of the plasmid genes in multiplex reactions. Although the use of the FAM-labelled probe would help increase the specificity of the assay, a positive reaction would not actually indicate which plasmid gene was present in this case as all probes were labelled with a FAM dye. It was also interesting to note that the authors still reported the presence of non-specific amplification from the formation of primer-dimers. Later, using SYBR Green PCR and melting curve analysis, Geyer and Hanson (2014) published a method for the detection and differentiation of AmpC plasmid

genes in a single multiplex assay, based on the melting temperature of the PCR product. They reported an assay performance of 100% sensitivity and 96% specificity for a range of plasmid genes and in a range of different Enterobacteriaceae species tested.

Boot *et al.* (2013) developed a SYBR Green and melting curve protocol for the detection of ESBL genes carried on plasmids in *E. coli*. They observed a ten-fold increase in sensitivity of the real-time PCR assay compared to using gel electrophoresis as the detection method. The intensity of the bands on the electrophoresis gel was reported to correspond with the height of the melting curve peaks. Chandramohan and Revell (2012) also used SYBR Green PCR and melting curve analysis for the detection of ESBL genes in Enterobacteriaceae.

The use of melting curve analysis in the current study facilitated the development of multiplex assays for the detection of AmpC plasmid genes. Each of the intended amplicons gave consistent melting temperatures for both simplex and multiplex assays. Although there was some overlap in the position of the peaks on the melting curve graphs, this did not present a problem as the multiplex assays were intended to be used as an initial screen, with subsequent confirmation using simplex PCR assays with individual primer sets. Furthermore, it was not expected that isolates would possess multiple plasmid genes; so only one peak would usually be present for each assay.

3.4.2 Updated Primers for AmpC Plasmid Gene Groups

In this study, the original primers for the six AmpC plasmid gene groups were reviewed for current suitability. It was not surprising to note that some of the primer pairs would no longer detect all the AmpC plasmid genes currently recorded. However, it could be argued that with the predominance of CMY-2 and DHA-1 as the most frequently encountered plasmid-mediated AmpC enzymes in the UK (Woodford *et al.*, 2007), the need to fully encompass all current plasmid genes may not be necessary. However, the counter-argument that emerging plasmid genes would not be detected unless regular reviews of primer sequences are undertaken also holds true. Although commercial diagnostic assays are available for the

detection of AmpC plasmid genes, they are more expensive than the traditional culture-based methods. In the clinical setting, there is a balance to be struck between speed, accuracy and utility and the price of the assay. The benefit of using culture as a screening method for resistant strains is that new, emerging mechanisms should still be detected.

Both the CMY-2 and DHA-1 plasmid genes would be detected using the primers published in the original paper (Perez-Perez & Hanson, 2002), representing the CIT and DHA plasmid gene groups, respectively. The review did highlight the speed at which additional plasmid genes are being detected and recorded. Any review of this nature will only have a limited lifespan if full coverage is a key objective. Indeed, it was not even possible to achieve full coverage for some of the plasmid gene groups. The CMY-13 gene had a large number of additional nucleotides present, making alignment with other members of the CIT group impossible. This sequence did not align with any of the other five plasmid gene groups either. In the case of the ACC group, it was not possible to identify new primers for the four plasmid genes recorded. The two additional ACC plasmid genes (ACC-3 and ACC-4) have not yet been reported to be present in the UK (Girlich *et al.*, 2000; Papagiannitsis *et al.*, 2007). The other four AmpC plasmid gene groups were either covered by existing primers (DHA) or were able to have updated primers selected from aligned sequences (FOX, MOX, EBC). Although some other studies have also reported the use of updated primers for plasmid-mediated *ampC* genes (Dallenne *et al.*, 2010; Voets *et al.*, 2011), these were also found not to fully include all plasmid genes available at the time of this review (data not shown).

3.4.3 Further Developments

One obvious future development would be the use of probes labelled with different fluorescent dyes. This would enable the detection of the AmpC plasmid genes, with a simultaneous identification of the plasmid gene group present through the use of different coloured-dyes. One limitation of this approach would be the number of detection channels available on the real-time PCR instrument. In the case of the Smart Cycler instrument used in this study, there are four detection channels

available. This would limit the number of target probes to three plasmid gene groups if one channel was allocated for an internal control (Public Health England, 2013).

The assays developed in this stage of the study were subsequently used for the detection of *bla*$_{AmpC}$ and *bla*$_{CTX-M}$ genes present in local and regional isolates of *E. coli*. Although the AmpC plasmid primers were reviewed against the current list of known plasmid genes, the same process was not undertaken for the CTX-M genes. At the time of last checking, there were 172 CTX-M genes listed on the international website (www.lahey.org/studies/other, last accessed 15th July 2015). It is likely that many of these will have been discovered since the CTX-M primers used in this study were first developed. It would be useful as a future study to investigate whether the universal CTX-M primers and Group 1-specific CTX-M primers are still able to detect the clinically relevant CTX-M genes. Although many studies report CTX-M-15 as the most common type of ESBL present in *E. coli*, there is a danger that the use of primers that are now over ten years old will, in time, miss some of the newer genes as they arise. It is worth noting that CTX-M ESBL enzymes were only discovered 15 years ago (Livermore, 2007).

The underlying PCR protocols in the study were also used for the amplification of target genes prior to sequencing. The use of the melting curve analysis was helpful in identifying the successful amplification of a product before the sample was referred for sequencing. Although not investigated further, there was some anecdotal evidence that the height of the melting curve peak was related to the success of achieving a full sequence result. Therefore, a judgement could be made on whether to repeat an amplification step before sending the product for sequencing.

4 Pilot Study of AmpC-Mediated Resistance in Gloucestershire

4.1 Introduction

All *E. coli* strains carry the chromosomal *ampC* gene for enzyme production (Olsson *et al.*, 1983). This gene is expressed weakly, but constitutively, and at a level low enough that clinical resistance to beta-lactam antibiotics is not seen. Mutations can, however, occur in the promoter region of the gene, affecting the expression and subsequent production of the AmpC enzyme. Strains with these mutations are said to hyper-produce the AmpC enzyme (Caroff *et al.*, 2000).

The common RNA promoter sequence for *E. coli* includes a -35 box (TTGACA), separated from a -10 box (TATAAT) by a distance of 17 base pairs (Hawley & McClure, 1983). The promoter sequence for the *ampC* gene, however, is different by a single base pair change in each of the boxes, and has an inter-box distance of 16 base pairs. It is these small changes that reduce the power of the promoter and the level of enzyme expression (Corvec *et al.*, 2002).

There are reported mutations that can return the wild-type promoter sequence to one closer to the common promoter sequence for *E. coli*. The mutations create either a new -35 box in a different location, or change the existing -35 box. The most commonly reported mutation is a C to T transition at position -42 in the promoter region (Tracz *et al.*, 2007). This mutation creates a displaced -35 box, increasing enzyme production by 20-fold when compared to the wild-type level. Other important mutations affecting enzyme expression include a T to A transition at position -32 and the insertion of one or two bases at position -13 (Tracz *et al.*, 2007). Mutations have also been reported throughout the rest of the promoter and attenuator regions, but have less of an impact on enzyme expression. Mulvey *et al*. (2005) found 91% of 183 cefoxitin-resistant *E. coli* isolates had one or more mutations at 29 different locations in the sequenced *ampC* promoter regions.

E. coli strains can also acquire *ampC* genes on plasmids from other species. Plasmid *ampC* genes were first described in 1988, and are derived from species such as *Enterobacter* spp. and *Citrobacter* spp. (Philippon, Arlet & Jacoby, 2002). AmpC

plasmid genes have been classified into six groups, based on the species of origin: CIT, ACC, DHA, FOX, MOX and EBC (Perez-Perez & Hanson, 2002). The most common AmpC plasmid gene, also with the largest geographic spread, is the CMY-2 gene (CIT-type) (Jacoby, 2009).

In a UK study (Woodford *et al.*, 2007) of cephalosporin-resistant *E. coli*, 49% had an AmpC plasmid-mediated gene present. The isolates were submitted to the national UK reference laboratory, and were selected for potential AmpC production based on the presence of cephalosporin-resistance without potentiation by clavulanic acid. The majority of isolates with plasmid genes carried the CIT-type, but ACC, FOX and DHA plasmid gene groups were also found.

Even though *E. coli* is one of the most common pathogens in the UK, there is a lack of data for AmpC resistance in clinical isolates. This is particularly the case for the chromosomal mutations in the *ampC* promoter region. In a study by a national UK reference laboratory (Hopkins *et al.*, 2006a), 12 isolates were found with mutations in the *ampC* promoter region. These isolates were from an archived library, were collected between 1995 and 2003, and are unlikely to be fully representative of current clinical strains.

Epidemiological studies are only ever a snapshot of a particular time and place, so it is important to update and renew the data wherever possible. This helps to ensure that the rapid spread of new resistance genes or dominant clones are detected quickly. The CTX-M ESBL enzymes, for example, went from being first reported in 2000 to the predominant cause of cephalosporin resistance in the UK in less than 5 years (Livermore *et al.*, 2007).

This stage of the current study was considered to be a pilot study for the larger, regional study. Undertaken in 2011, with isolates collected from clinical urine samples in the Gloucester laboratory, the purpose of the pilot study was to establish the background data for prevalence of AmpC plasmid genes and *ampC* chromosomal promoter region mutations, and to evaluate the PCR assays and

protocols for sequencing products. This phase of the study has been published in the British Journal of Biomedical Science (Lewis *et al.*, 2015).

4.2 Methods

Isolates were collected in the Gloucester laboratory during the period June 2011 and November 2011, and were eligible for inclusion in the study if they had a phenotypic susceptibility pattern suggestive of AmpC enzyme production; resistant to cefpodoxime, but with a negative clavulanic acid synergy test (M'Zali *et al.*, 2000). Isolates were excluded if the same species had been isolated from the same patient in the previous 28 days. Isolates were allocated a unique study reference number, in order to anonymise patient-specific data. Isolates were collected from samples referred from both hospital and community settings. As samples from GP surgeries represent only a subset of actual UTI cases, this may have introduced an element of bias into the collection process (McNulty *et al.*, 2006).

Isolates were identified using chromogenic agar plate media and biochemical identification strips. Disc susceptibility testing was performed according to a national standard method (Andrews & Howe, 2011) to a range of cephalosporins, including cefpodoxime, cefuroxime, cefoxitin, cefotaxime and cefepime. Isolates were tested to determine the MIC of cefotaxime, using gradient antibiotic strips, and were also tested for the presence of ESBL enzymes using clavulanic acid synergy discs.

DNA templates for PCR were prepared using a crude lysis method (Woodford, 2010). The presence of *ampC*-carrying plasmids was determined using two multiplex real-time SYBR Green PCR assays. The primers used were a combination of those published previously (Perez-Perez & Hanson, 2002) and those developed in this study (Section 3.3.6). The multiplex assays were designed to detect CIT, ACC and DHA plasmid gene groups in one assay, and the FOX, MOX and EBC plasmid gene groups in the other. Multiplex AmpC plasmid gene PCR assays with a positive melting curve peak were confirmed using the same primers in three separate simplex reactions.

A 271bp region of the *ampC* gene was amplified and sequenced, using primers previously published by Caroff *et al*. (2000). The amplified sequence included the promoter region, attenuator region and the first part of the coding region. The PCR products were sent to an external laboratory (Eurofins Genomics, Germany) for sequencing. The reported sequence of each product was compared against the *ampC* sequence for the *E. coli* NCTC 12241 strain (GenBank Accession No. AY899338).

4.3 Results

4.3.1 Patient Demographics

A total of 50 *E. coli* isolates were isolated from urine samples sent in for clinical diagnostic purposes. A total of 75% of isolates were from female patients and 25% were from male. The mean age of all patients was 58.3 years, with a range from 1 to 94 years. No further demographic data were collected on the samples.

4.3.2 Susceptibility Testing

All isolates were tested against a range of five cephalosporins, with results analysed using the breakpoints set by the British Society of Antimicrobial Chemotherapy (BASC) (Andrews & Howe, 2011). The summary of results is given in **Table 4-1**.

The majority of isolates (98%) were resistant to cefuroxime at the breakpoint of a 19mm zone size. This is in contrast to the cefotaxime results, in which 28% of isolates were resistant at the 19mm breakpoint zone size. Further results included 98% of isolates demonstrating resistance to cefoxitin and 44% with intermediate or resistant zone sizes for cefepime.

Isolates were also tested for the minimum inhibitory concentration of cefotaxime using gradient antibiotic strips. The geometric mean MIC for cefotaxime was 1.2mg/L for the whole group of 50 isolates, which is just above the recommended clinical breakpoint of 1.0mg/L (Andrews & Howe, 2011).

Table 4-1: Disc susceptibility testing results for the 50 pilot study isolates of *E. coli* to a range of five cephalosporins.
The zone diameter breakpoints shown are for resistant isolates, or for resistant and intermediate isolates if a range is given. S = Resistant, I = Intermediate, R = Resistant. Percentage values are given in parenthesis. (Andrews & Howe, 2011).

	Disc Content (µg)	Zone Diameter Breakpoints (mm)	S (%)	I (%)	R (%)
Cefpodoxime	10	≤19	0 (0)	N/A	50 (100)
Cefuroxime	30	≤19	1 (2)	N/A	49 (98)
Cefotaxime	30	≤23 to ≤29	14 (28)	22 (44)	14 (28)
Cefoxitin	30	≤22	1 (2)	N/A	49 (98)
Cefepime	30	≤26 to ≤31	28 (56)	12 (24)	10 (20)

4.3.3 Prevalence of *ampC*-Carrying Plasmids

The 50 isolates were tested for the presence of AmpC plasmid genes using the two multiplex AmpC plasmid gene PCR assays (CIT/ACC/DHA and FOX/MOX/EBC) developed in Section 3.3.6. Ten of the 50 isolates gave an initial positive result in the CIT/ACC/DHA multiplex assay (**Table 4-2**). All of these were subsequently confirmed as being a CIT-type gene using the simplex version of the assay (**Figure 4-1**). None of the other AmpC plasmid gene groups were detected (ACC, DHA, FOX, MOX, EBC). The isolates with the CIT-type genes were not investigated further to determine the specific plasmid gene present.

Table 4-2: Melting temperatures (TM) from the multiplex AmpC plasmid gene assay for the detection of CIT/ACC/DHA plasmid genes in *E. coli*, with the corresponding repeat simplex assay for confirmation.
Also shown are the cycle threshold (CT) values for the simplex PCR reactions using the CIT primers.

Isolate	Multiplex TM (^{o}C)	Simplex CT	Simplex TM (^{o}C)
G-010	88.8	22.6	88.6
G-012	88.9	19.5	88.6
G-014	88.6	26.3	89.5
G-017	88.6	16.9	88.8
G-018	88.9	22.4	89.0
G-026	88.5	23.1	88.7
G-042	88.5	25.6	88.7
G-050	88.8	22.9	89.0
G-052	88.7	21.5	88.8
G-054	88.7	26.3	88.2

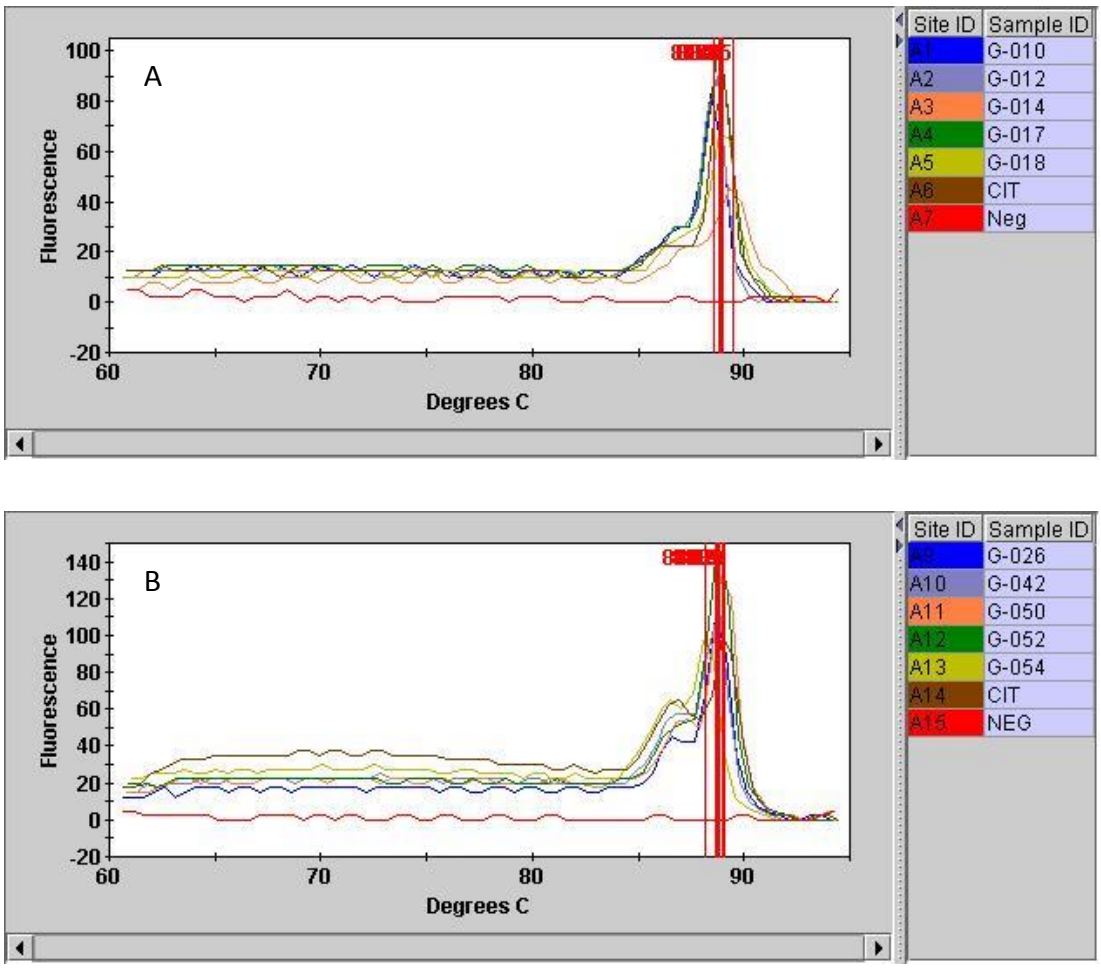


Figure 4-1: Melting curve graphs of the ten isolates identified as carrying a CIT-type AmpC plasmid gene.
The assays were simplex PCR reactions to confirm the presence of a positive peak in the screening multiplex assays. Each of the two sets of five simplex reactions was tested along with a bacterial control strain (CIT) and a negative PCR control (NEG) of water. Isolates were tested in two batches; A and B.

4.3.4 *ampC* Promoter Region Mutations

In the remaining 40 isolates (those without an AmpC-plasmid gene present), sequencing the 271bp region of the chromosomal *ampC* promoter revealed mutations at 16 different locations. The full list of mutations is provided in **Table 4-3**.

From the 16 mutations, a total of 18 different genotype patterns were recorded. All of the 40 isolates had at least one mutation in the amplified sequence, when compared to the sequence for the *E. coli* NCTC 12241 control strain (GenBank No: AY899338). Genotype numbers were allocated to each of the unique patterns,

based initially on the frequency of detection (e.g. G1 most common, G2 second most common, etc.).

In general, the types of mutations detected fell into three principal groups:

1. Mutations in the wild-type promoter region (Section 4.3.4.1)
2. Creation of a displaced promoter (Section 4.3.4.2)
3. Increases to the spacer region distance (Section 4.3.4.3)

4.3.4.1 Mutations in the Wild-Type Promoter

The most common mutation detected in the 40 isolates was a T to A substitution at position -32. This mutation was found in a total of 25 isolates (62.5%) overall, but was also commonly associated with other mutations at positions +58 and +63. This genotype pattern of -32, +58, +63 (designated G1) was found in a total of 11 isolates, and represented the most common genotype pattern detected. The -32 mutation alters the normal wild-type -35 box from TTGTCA to TTGACA, thus retaining its original position but now with a promoter sequence identical to that of the *E. coli* consensus promoter (TTGACA) (Hawley & McClure, 1983).

Table 4-3: Details of mutations observed at different positions in the amplified 271bp region of the promoter, attenuator and coding regions of the chromosomal *ampC* gene. Each genotype is shown, with the corresponding numbers of isolates allocated to that group. Genotype numbers were allocated within this study. Position numbers for locations on the *ampC* gene were those used by Jaurin *et al.* (1981). The control sequence is derived from the GenBank entry for *E. coli* NCTC 12241 (AY899338). ins = 1 or 2 bp insertions at position -13.

Genotype	No. of Isolates	-42	-32	-28	-18	-14	-13	-13ins	-1	+6	+17	+23	+34	+37	+58	+63	+70	+81
Control		C	T	G	G	T	T	-	C	C	C	G	G	G	C	T	C	A
G1	11		A												T	C		
G2	8		A															
G3	2		A														T	G
G4	2							T		T								
G5	2	T			A				T						T			G
G6	2			A				AT										
G7	2			A		A		T			T							
G8	1				A				T						T			G
G9	1			A														
G10	1			A				T										
G11	1						G	TT										
G12	1		A	A							T							
G13	1		A	A														
G14	1		A		A				T			A			T			G
G15	1		A											A			T	G
G16	1	T			A				T			A			T			G
G17	1			A									A		T			
G18	1			A							T							

The -32 mutation was also found with mutations at other positions in the amplified sequence. Two isolates had mutations at positions +70 and +81 in the coding region, in addition to the change at position -32 (Genotype G3). One isolate had mutations at positions -32 and -28 (Genotype G13), and one isolate had mutations at positions -32, -28 and +17 (Genotype G12). Two further isolates had the -32 mutation present with a number of various changes at other positions in the region (-18, -1, +23, +37, +70 and +81). The -32 mutation was also detected as the only nucleotide change present. This was observed in a total of 8 isolates (20%), and was allocated the genotype number G2, as the second most common genotype.

4.3.4.2 Creation of a Displaced Promoter

Mutations at positions -42 and -18 have been reported to create new -35 and -10 boxes, respectively, at different locations in the promoter region (Olsson *et al.*, 1983). Of the 40 isolates tested, the -42 mutation (C to T) was found in only 3 isolates (7.5%). In all three cases, this mutation was found together with a G to A transition at position -18 (Genotypes G5 and G16).

The -18 mutation itself was found to be present in two isolates without the -42 mutation. One of these isolates was found to have mutations at positions -18, -1, +58 and +81 (Genotype G8). In the other isolate, it was found to be present in conjunction with the -32 and other mutations (Genotype G14).

4.3.4.3 Increase in Spacer Region

The distance between the -35 and -10 boxes, either in the wild-type or the displaced locations, is known to have an influence on the level of enzyme production (Ayers *et al*., 1989). An increase in the spacer region can occur through the -42 and -18 mutations described above. The two mutations together create two new -35 and -10 boxes that are 17bp apart in the promoter region. The normal wild-type distance for the two boxes is 16bp.

However, increases in the spacer region were also observed in 8 of the 40 isolates (20%), with the insertion of one or two nucleotides at position -13. The additional bases were either adenine or thymine, and were found as single insertions in 5

isolates (Genotypes G4, G7, and G10) and double insertions in 3 isolates (Genotypes G6 and G11). In all 8 isolates, the insertions at position -13 were present along with mutations at other various positions, but not with either the -42 or -32 mutations.

4.3.4.4 Mutations at Other Positions

In addition to the mutations described above (-42, -32, -18 and -13ins), there were other mutations noted to be present in the 40 isolates. A mutation at position -28 (G to A) was observed in 25% of the isolates. This was associated with the -32 mutation (2 isolates) or with the -13 insertions (5 isolates), but not with the -42 mutation. In one isolate, the -28 mutation was the only mutation detected throughout the amplified region. In two isolates, it was found only along with mutations in the attenuator region (positions +17 and +34).

Mutations in the attenuator region (positions +17 to +37) are thought to destabilise the effect of the stem-loop and thereby allow for increased levels of enzyme transcription (Caroff *et al.*, 2000). In the 40 isolates tested, mutations in the attenuator region were observed in a total of 8 isolates, but always along with one of the key mutations (-42, -32 and -13ins) described by Tracz *et al.* (2007). In two isolates, this was seen in conjunction with a single base pair insertion at position -13. In three isolates, the attenuator mutations were observed along with the -32 mutation, and with the -42 mutation in one further case. As mentioned above, there were two further isolates in which the attenuator mutations were found together with the mutation at position -28.

Mutations were seen at three positions in the coding region (+63, +70 and +81). In total, 47.5% of isolates (n=19) had at least one of the mutations at these positions. They were seen in conjunction with both the -42 and the -32 mutations, but not the -13 insertions.

Mutations were also observed at three positions outside the promoter, attenuator and coding regions. Positions -1, +6 and +58 are outside the three main regions, but were commonly seen as mutations in the 40 isolates tested. Two of the mutations (-1 and +58) were occasionally found together, as was the case in 5 of the 40

isolates. In total, there were 24 of the 40 isolates in the group with mutations in at least one of these three positions.

4.3.5 Mutations Seen in the AmpC Plasmid Gene-Carrying Isolates

The ten isolates carrying a CIT-type AmpC plasmid gene were also investigated for the presence of mutations in the chromosomal promoter region. None of the ten isolates had one of the -42, -32 or -13ins mutations present (**Table 4-4**). Eight of the isolates had mutations present at one of the three locations in the coding region (+63, +70 or +81). The mutations at positions -28, -18 and -1 were each present in two of the ten isolates. The mutation at position +58 was present in three isolates.

Table 4-4: *ampC* chromosomal mutations present in the *E. coli* isolates carrying AmpC CIT-type plasmid genes.

Study Ref	AmpC Plasmid Gene	*ampC* chromosomal mutation(s)
G-010	CIT	+81
G-012	CIT	-18, -1, +58, +81
G-014	CIT	+70, +81
G-017	CIT	-28,
G-018	CIT	+70, +81
G-026	CIT	+70, +81
G-042	CIT	-18, -1, +58, +81
G-050	CIT	+58, +63
G-052	CIT	+81
G-054	CIT	-28

4.4 Discussion

4.4.1 Susceptibility Testing

The use of cefoxitin resistance as a screening method is recommended in national standard methods for susceptibility testing (Andrews & Howe, 2011). All but one of the isolates (98%) were resistant to cefoxitin by disc susceptibility testing, thus confirming its use as a screening method. The specificity of the method, however, is reduced by the existence of other means in which *E. coli* strains can become resistant to cefoxitin (i.e. membrane permeability) (Chen & Livermore, 1993).

Strains producing AmpC enzymes are normally considered to exhibit the antibiogram phenotype of cefoxitin resistance, but with cefepime susceptibility (Potz *et al.*, 2006; Woodford *et al.*, 2007). In this study, only 56% of the isolates met the criteria for both cefoxitin-resistant and cefepime-sensitive, as only 42% had a disc zone size larger than the 32mm breakpoint for cefepime susceptibility. This highlights the importance of using a method (phenotypic or molecular) to confirm initial antibiogram results.

4.4.2 Prevalence of *ampC*-Carrying Plasmids

The results from this pilot study of 50 isolates revealed chromosomal mutations and acquired genes associated with AmpC resistance. Ten of the isolates (20%) had a CIT-type plasmid gene present. Although not fully characterised in this study, the most common of the CIT-type plasmid-mediated genes is CMY-2; reported globally in clinical samples (Mammeri *et al.*, 2008; Jorgensen *et al.*, 2010; Bogaerts *et al.*, 2010; Mulvey *et al.*, 2005). It has also been associated with poultry (Voets *et al.*, 2013; Dhanji *et al.*, 2010), livestock and companion animals (Dierikx *et al.*, 2012), water and beaches (Mataseje *et al.*, 2009) and retail vegetables (van Hoek *et al.*, 2015b). The importance of monitoring AmpC plasmid genes in clinical samples is discussed further in Section 1.4, and was highlighted recently in a case report (Lin *et al.*, 2015). A CMY-2 plasmid gene was reported to have transferred from an *E. coli* strain to a *Klebsiella pneumoniae* strain isolated from a liver abscess. Both strains had been isolated from the same patient.

The presence of *ampC*-carrying plasmids in 20% of the cephalosporin-resistant *E. coli* isolates in the current study appears to be in line with studies from other countries. Mammeri *et al.* (2008) investigated 2800 isolates of *E. coli* in France, finding 34 with an AmpC phenotype. Most of the isolates were isolated from urine samples or purulent exudate. Eight (23.5%) had an AmpC plasmid gene present, the majority of which were CMY-2. In Denmark, Jorgensen *et al.* (2010) found 24 of 6356 *E. coli* isolates to have a phenotype suggestive of AmpC-mediated resistance. CIT-type plasmid genes were found in 4 of the 24 isolates (16.7%), all of which were confirmed by sequencing as CMY-2.

Bogaerts *et al.* (2010) investigated 83 *E. coli* isolates in Belgium with a confirmed AmpC phenotype. The isolates had been isolated mainly from urine samples or screening rectal swabs. Of the 83 isolates, 13.3% (n=11) carried an AmpC plasmid gene; ten isolates with CMY-2 and one isolate with ACC-1. Finally, in a Canadian study, Mulvey *et al*. (2005) reported 13.5% of mainly uropathogenic *E. coli* isolates to be carrying an AmpC plasmid gene. All of these were confirmed to be CMY-2.

4.4.3 *ampC* Promoter Region Mutations

In the 40 isolates without an AmpC plasmid gene present, 70% (n=28) possessed either the -42 or -32 mutation (**Figure 4-2**); both of which are considered to be key factors for increased AmpC enzyme production. These two mutations are known to create a new -35 box, to one with a greater degree of sequence identity to the *E. coli* consensus RNA promoter sequence (Tracz *et al.*, 2007).

The -32 mutation (T to A) was the most common mutation found, present in 62.5% of isolates without an AmpC plasmid gene present. The mutation was present either alone or in combination with mutations at other positions. First described by Kobayashi *et al*. in 1990 (Kobayashi, Nagata & Ishihama, 1990), the -32 mutation is reported to cause an increase of enzyme over-expression by up to 46-fold when compared to the susceptible wild-type *E. coli* strains (Tracz *et al.*, 2007). In the current study, the cefotaxime MIC tests gave a geometric mean of 0.6mg/l for the isolates with the -32 mutation present. This represented a 10-fold increase in the MIC when compared to the *E. coli* control strain (NCTC 12241) MIC of 0.06mg/l.

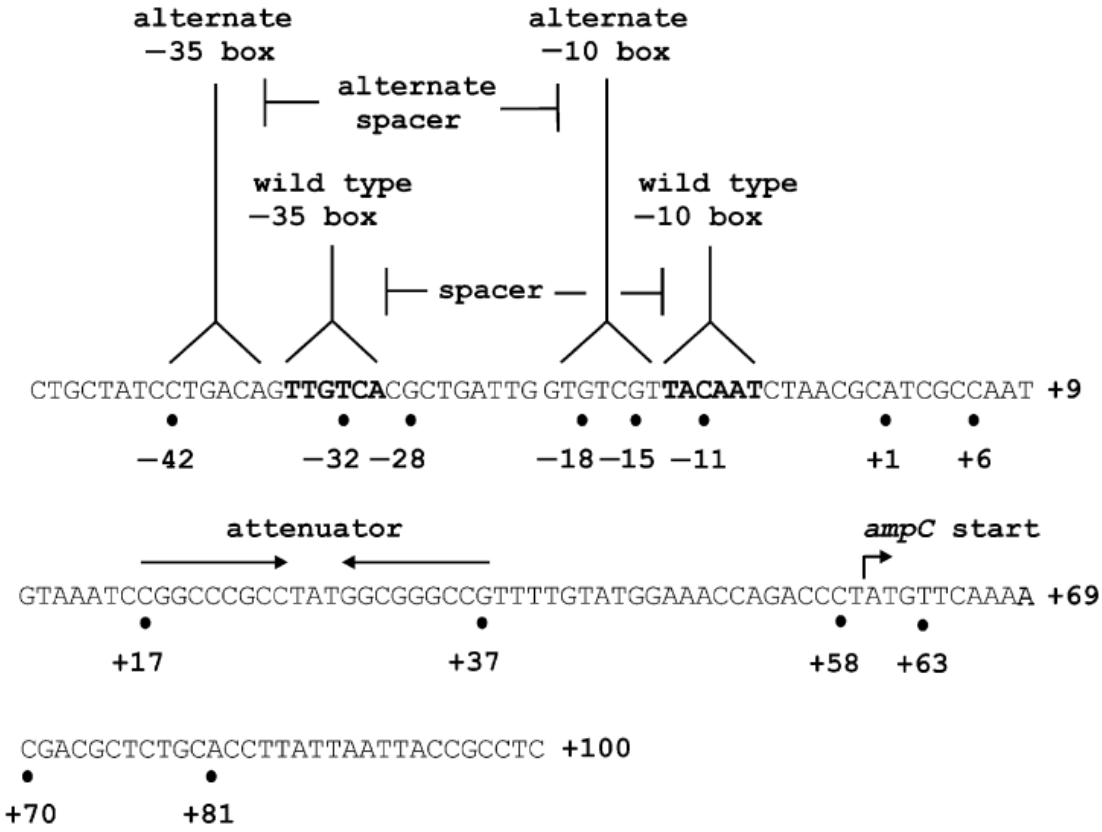


Figure 4-2: Sequence of the *E. coli* ATCC 25922 (NCTC 12241) *ampC* promoter region.
Reproduced with permission from Tracz *et al*. (2007).

Caroff *et al.* (2000) cloned sections of the *ampC* promoter region into indicator genes. The sections of the promoter region investigated included the -42 and -32 locations, tested both with and without mutations. Results indicated that enzyme concentrations increased by 70- to 120-fold in the presence of the mutations. Tracz *et al*. (2007) also found significant increases in promoter expression for the -42 mutation and -13 spacer insertions, of 20-fold and 24- to 61-fold, respectively.

In the current study, only three isolates (6%) possessed the -42 mutation. Other studies, however, have found a higher prevalence for this particular mutation. Mammeri *et al.* (2008) found 57.7% (n=15) of isolates in a French study with the -42 mutation and 23.1% (n=6) of isolates with the -32 mutation. In a Danish study, Jorgensen *et al.* (2010) found 25% (n=5) of isolates each with the -42 mutation and the -32 mutation, equally. Bogaerts *et al*. (2010) found that 100% (n=72) of the isolates in Belgium with chromosomal mutations had the -42 mutation present.

Mulvey *et al*. (2005) found 23.5% of isolates in the large Canadian study to have the -42 mutation present and 18.0% of isolates to have the -32 mutation. To date, the current study (Lewis *et al.*, 2015) appears to be the only published data in which the -32 mutation is more prevalent than the -42 mutation.

It is thought that the -42 and -32 mutations are able to have a direct effect on the transcription process, due to the binding of the RNA polymerase (Olsson *et al.*, 1983). In the current study, the -32 mutation was seen as the only mutation present in 20% of isolates (n=8), thus confirming its importance as a controlling influence on enzyme production.

Mutations were seen at positions -28 and -18 in ten isolates and five isolates, respectively. These were seen commonly in conjunction with the insertions at position -13, but were also observed together with the -32 mutations. Corvec *et al.* (2007) also noted that the -18 and -28 mutations were present in a number of the fully sensitive isolates they tested (13% and 29%, respectively), thus calling into doubt the role these two mutations actually play in influencing enzyme production. Tracz *et al.* (2007) demonstrated that the -18 mutation alone had a 1 to 4-fold increase in enzyme production and the -28 mutation a 1 to 3-fold increase, also confirming a minimal role in promoter control.

Ayers *et al.* (1989) first described the impact of the spacer region between the -35 and -10 boxes in 1989. Tracz *et al.* (2007) demonstrated that strains with a single base pair insertion at position -13 had a 24- to 61-fold increase in enzyme expression. This was similar to the effect seen with two nucleotides inserted at the same position. The insertion of one or two base pairs at position -13 was recorded in 20% of isolates tested in the current study. The inserted bases were either adenine or thymine, and increased the spacer region between the -35 and -10 boxes from 16bp to 17 or 18bp. Mulvey *et al.* (2005) reported 10.4% of isolates with the insertions at position -13 (1 or 2bp).

Mutations in the attenuator region (positions +17 to +37) were found in 17.5% of isolates (n=7). These mutations were thought to increase enzyme production

through the destabilisation of the stem-loop structure (Caroff *et al.*, 2000), but have been demonstrated to have little actual effect on enzyme production (Tracz *et al.*, 2007). Mulvey *et al.* (2005) described a small number of isolates with single base pair deletions at a position in the attenuator region (positions +34 and +37), but these were not seen in the current study. Evidence for the impact of attenuator mutations is somewhat indirect in nature, and is currently limited to the functional assays described above. Introducing mutations into the attenuator region to obtain direct evidence is considered to be difficult due to the stem-loop structure (Caroff *et al.*, 2000). Mutations in the coding region (positions +63 to +81) were seen in nearly half (47.5%) of all isolates. The contribution of these mutations in unknown (Mulvey *et al.*, 2005).

4.5 Summary

The pilot stage of the project tested 50 isolates of uropathogenic cefpodoxime-resistant, ESBL-negative *E. coli*. AmpC-mediated resistance was confirmed in 92% of isolates. Although some isolates did carry a plasmid-mediated *ampC* gene, the majority possessed one of the key *ampC* chromosomal promoter region mutations (-42, -32, -13ins) known to cause hyper-production of the enzyme (Tracz *et al.*, 2007).

The -32 mutation, -42 mutation and -13 insertions accounted for 90% of the resistance in the 40 isolates without an AmpC plasmid gene present. These three key mutations were not found together in the same isolate, providing further evidence that they are the primary chromosomal mechanisms responsible for enzyme hyper-production. Indeed, the -32 mutation was seen as the only mutation present in a fifth of isolates.

There is a general lack of published data for the prevalence of these key mutations within UK strains. Hopkins *et al*. (2006a) investigated a selection of isolates that had been referred to the Laboratory of Enteric Pathogens, London. Of the 37 archive isolates tested, 12 had chromosomal mutations potentially related to AmpC hyper-production, but the -42 and -32 mutations were only found in half.

In the current study, the -32 mutation was the most prevalent, rather than the -42 mutation reported as the most common elsewhere. It is unclear whether the two mutations would have significantly different impacts on actual clinical resistance. The studies by Tracz *et al.* (2007) indicated that the -32 mutation resulted in a 10 to 45-fold increase in enzyme production, whereas the isolates with the -42 mutation had 22 to 280-fold increases. Whether this would actually translate to a difference in the MIC for the isolate is not clear. As it is unlikely that the two mutations have significantly different effects on the level of enzyme production, this probably does not represent a clinically important difference. However, the difference may be a reflection of the variation in dominant clones in the local strain population. The extension of the project to include regional isolates, and to use molecular typing methods, will help to confirm whether this pattern is observed in other areas.

4.6 Future Work

The typing of the isolates with different mutations could give an indication of whether a dominant clone exists within the local population. This may explain why the isolates with the -32 mutation were more prevalent in this study. Using a PCR-based phylogenetic method (Clermont, Bonacorsi & Bingen, 2000), *E. coli* isolates can be allocated to one of four phylogenetic groups (A, B1, B2 and D), based on the presence of three PCR products (two genes *chuA* and *yjaA* and an anonymous fragment TSPE4). Pathogenic extra-intestinal strains are often reported to be in Groups B2 or D, whereas the environmental commensal strains are often in Groups A and B1. Typing methods were not used in the pilot stage of the study. However, an assay for phylogenetic typing the *E. coli* isolates would be a reasonably easy development using the SYBR Green PCR protocols.

Corvec *et al*. (2007) found a range of *ampC* chromosomal mutations in fully-sensitive isolates of *E. coli*. Of 100 isolates tested, 78 had mutations present at positions that included -88, -82, -73, -28, -18, -1, +17, +58, +63, +70 and +81. It is interesting to note that none of the three key mutations (-42, -32 and -13ins) described by Tracz *et al*. (2007) were found in the sensitive isolates. The current pilot study did not include the investigation of sensitive isolates or isolates with

other resistance phenotypes (e.g. ESBLs). However, this could be one useful area for investigation, to provide further evidence for the actual role of different mutations in the degree of phenotypic resistance observed.

5 Study of Cephalosporin-Resistance in *E. coli* in South West England

5.1 Introduction

A number of previous studies have investigated the prevalence of bla_{ESBL} genes in the UK. One principal study was by Potz *et al*. (2006), in which 16 laboratories in London and the South East of England submitted a range of cephalosporin-resistant Enterobacteriaceae isolates. Isolates were categorised as: ESBL-producing (CTX-M), ESBL-producing (Non-CTX-M), AmpC-producing and a fourth group for other mechanisms. Although the presence of $bla_{CTX\text{-}M}$ genes was determined using PCR, the categorisation of AmpC-producing isolates was done on the basis of interpretive reading from the MIC data and species identification. The investigation of chromosomal *ampC* promoter mutations was not undertaken. However, the study did highlight a variation in the proportion of CTX-M-producing isolates of 0.6% to 4.3% between some of the laboratories and their catchment populations.

In an earlier study, Woodford *et al*. (2004) investigated the presence of $bla_{CTX\text{-}M}$ genes in 291 *E. coli* isolates from 42 laboratories across the UK. Rather than a planned submission, these clinical isolates were referred to the testing laboratory to confirm unusual resistance phenotypes. Although they would have represented a wide geographic spread of locations, the collection of isolates was bias towards a more resistant phenotype. The Group 1-specific $bla_{CTX\text{-}M}$ genes were the most common, at 96% of the isolates investigated.

Later, the same group (Woodford *et al.*, 2007) reported on a study of 135 *E. coli* and 38 *Klebsiella* sp. isolates that had been sent to the national reference laboratory for investigation of cephalosporin resistance (without evidence of ESBL production). Although again not a regional study as such, the study did include isolates from 86 laboratories across UK and Ireland. Nearly half of all the *E. coli* isolates were shown to have an acquired *ampC* gene present, with a variety of types found. This study still represents the most comprehensive UK study of AmpC plasmid gene carriage in *E. coli*.

The first study of AmpC plasmid gene carriage in UK isolates had actually been published the previous year (Hopkins *et al.*, 2006a). A total of 24.3% (n=25) of *E. coli* isolates had an AmpC plasmid gene present, all of which were of the CIT-type. The majority of these were subsequently confirmed as being CMY-7 (n=20). The isolates had been sent from 11 different laboratories in the UK. This study also investigated the presence of chromosomal *ampC* promoter mutations in 12 isolates that were cefoxitin-resistant but without an AmpC plasmid gene. The number of isolates tested was quite small, making interpretation of the significance difficult, but the -42 mutation was seen to be present in higher numbers than the -32 mutation (4 vs 2).

Data published from studies conducted in other countries are often limited to isolates collected in just one location. This is the case for studies in France (Mammeri *et al.*, 2008), Denmark (Jorgensen *et al.*, 2010), Spain (Gude *et al.*, 2013) China (Li *et al.*, 2008) and the United States (Coudron, Moland & Thomson, 2000). The principal large-scale overseas study is one in Canada (Mulvey *et al.*, 2005), in which 232 cefoxitin-resistant *E. coli* isolates were collected from 12 laboratories over a 1-year period in 2000. A total of 49 different *ampC* phylogroups were reported from mutations at 28 different locations in the promoter sequence.

The regional stage of the current study followed on from the initial pilot phase conducted in 2011. The principal aim was to see whether the pilot findings (Chapter 4) were mirrored elsewhere in the region, and to determine whether there were variations between laboratories for resistance rates and the types of genes present. This had been noted by Potz *et al*. (2006). In particular, one aim was to see whether the -32 mutation observed to be the most common in the pilot study was also the most common in isolates collected from other laboratories.

5.2 Methods

A full description of methods is provided in Chapter 2. Clinical isolates of cefpodoxime-resistant *E. coli* were collected from five laboratories in the South West region over a 3-month period. Isolates were excluded if the same species had been isolated from the same patient in the previous 28 days. Isolates were

identified using chromogenic agar plate media and biochemical identification strips, as described in Chapter 2.10. Isolates were allocated to an ESBL group or a Non-ESBL group, based on the results of a clavulanic acid synergy test (M'Zali *et al.*, 2000). The methods used for detecting $bla_{CTX\text{-}M}$, bla_{AmpC}, chromosomal *ampC* mutations and multi-locus sequence typing is provided in **Figure 5-1**.

5.3 Results

5.3.1 Isolate Collection

The regional phase of the current study was undertaken in 2013. Five laboratories were recruited to participate, from a total of 14 available in the South West region. Each laboratory was asked to submit the first 50 isolates isolated from the start of the collection period (April 2013). The Gloucester laboratory was asked to collect the first 100 isolates (in addition to the 50 isolates in the pilot study; Chapter 4).

The five laboratories collected a total of 310 isolates over a 3-month period. On receipt in the testing laboratory (Gloucester), all isolates were tested for confirmation of *E. coli* identification and for resistance to cefpodoxime. As there is known variation in the performance of susceptibility testing between laboratories (Livermore *et al.*, 2012), the mean zone sizes for cefpodoxime were checked for the isolates later confirmed to have *ampC* chromosomal mutations (**Table 5-1**). With the exception of Dorchester, all laboratories had mean zone sizes for cefpodoxime that were similar to the mean for the whole group.

Table 5-1: Mean zone sizes for cefpodoxime (10µg disc) for the 53 *E. coli* isolates with confirmed *ampC* chromosomal mutations.

Laboratory	No. of Isolates	Mean zone size (mm)	SD	P
Dorchester	5	7.4	0.55	0.029
Gloucester	23	10.0	1.87	1.000
Swindon	10	10.3	3.09	0.743
Taunton	8	10.1	2.75	0.919
Truro	7	10.9	3.72	0.411
Total	53	10.0	2.56	

SD; standard deviation

P values were calculated using unpaired t-tests

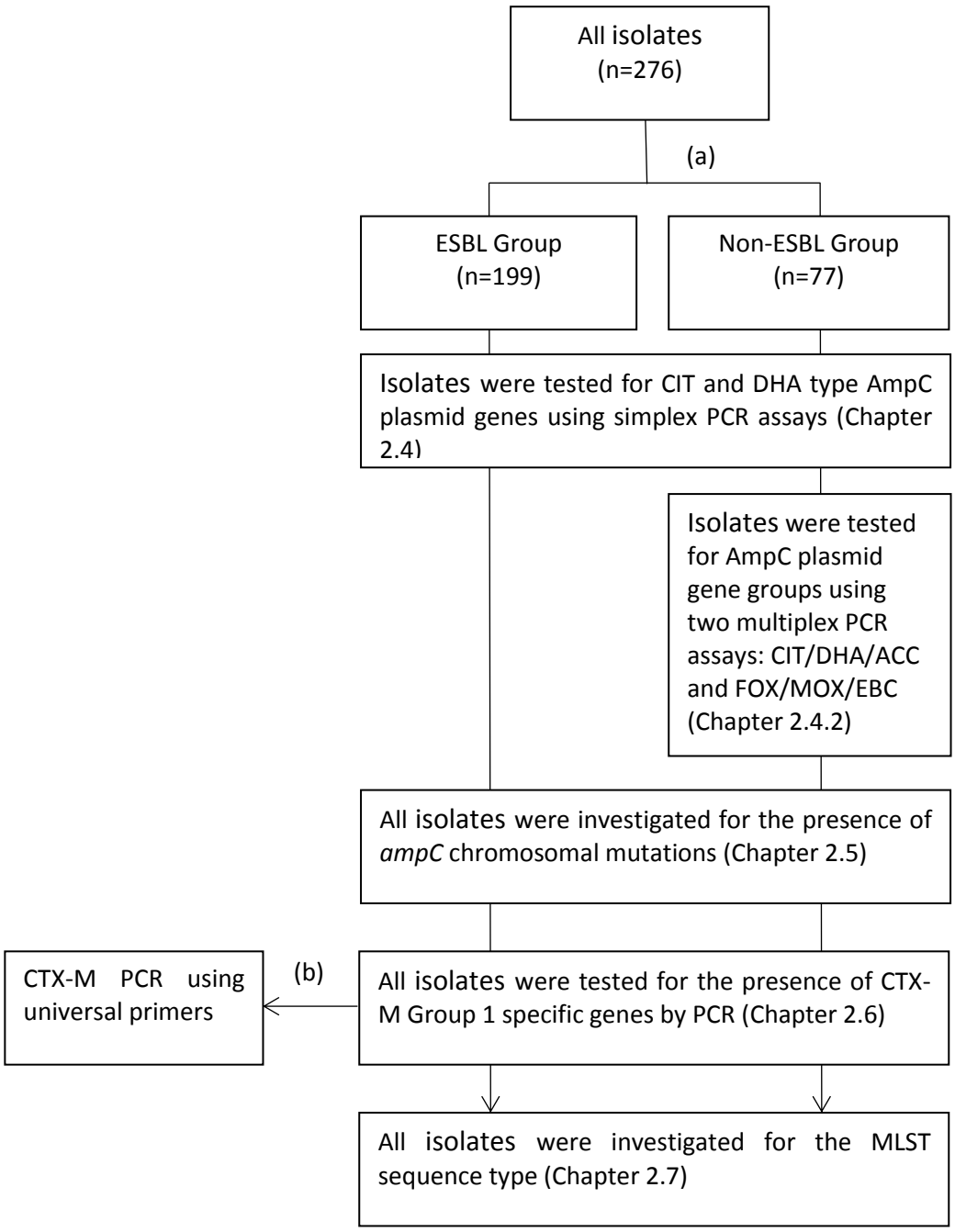


Figure 5-1: Sequence of methods used for detecting various resistance genes, *ampC* promoter mutations and assigning MLST-derived sequence types.

The relevant sections in Chapter 2 for the description of methods are provided in parentheses.

(a) Isolates were allocated into an ESBL or Non-ESBL using a clavulanic acid synergy test

(b) Isolates that were negative for the presence of CTX-M Group 1 genes were tested using the CTX-M universal primers

From the initial screening tests, a total of 35 isolates were excluded from the study: 10 isolates were susceptible to cefpodoxime; 20 isolates were species other than *E. coli* (see Appendices, Section 9.9); 4 isolates were removed as potential duplicates of other isolates; and one isolate was submitted without demographic data.

There were 276 isolates finally eligible for inclusion in the study. Confirmation of ESBL enzyme production by synergy discs allowed the isolates to be allocated to either an ESBL-producing group or a Non-ESBL group (**Table 5-2**), based on the reversal of cefpodoxime resistance in the presence of clavulanic acid. For practical purposes, the Non-ESBL Group of isolates was considered to be the group of isolates most likely to have potential AmpC mechanisms present. This was supported by the fact that 92% of the isolates in the pilot study (all cefpodoxime-resistant and ESBL-negative) were shown to have either an AmpC plasmid gene present or one of the key *ampC* chromosomal mutations (-42, -32 and -13ins) described by Tracz *et al*. (2007).

Table 5-2: Details of the numbers of isolates submitted from each laboratory; together with the numbers of isolates excluded.
Isolates were excluded on the basis of species identification, cefpodoxime susceptibility, potential duplicates and lack of demographic data. Isolates were allocated to either the ESBL or Non-ESBL group, based on the results of phenotypic cefpodoxime / clavulanic acid synergy tests.

Laboratory	No. Submitted	No. Excluded	ESBL Group	Non-ESBL Group
Dorchester	51	5	29	17
Gloucester	108	17	63	28
Swindon	50	0	36	14
Taunton	51	7	31	13
Truro	50	5	38	7
Total	**310**	**34**	**197**	**79**

5.3.2 Demographic Data

Of the 276 isolates, the majority were isolated from urine samples (97.1%). Other sample types included blood cultures (n=6) and wound swabs (n=2). Isolates from female patients were most common (74.6%), and isolates from GP surgeries made up the majority (76.1%) of source locations. The mean age of patients was 66.7 years, with a range of 1 to 102 years. The breakdown of how the demographic data varied by laboratories is given in **Table 5-3**. It was interesting to note that the Swindon laboratory had a higher proportion of female patients (92%) compared to the average, but a lower proportion of GP samples. The proportion of female patients was statistically significant ($p<0.0001$) using the chi-square test. However, the proportion of GP patients was not found to be significant ($p=0.06$), when compared against the mean.

Table 5-3: Distribution of patient demographic data between the five laboratories.
The proportions of female patients and samples originating from GP patients are given as percentages. The mean age is calculated from the ages of patients (in whole years) at the time the sample was collected.

Lab	Female Patients (%)	GP Samples (%)	Mean Age (years)
Dorchester	67.4	84.8	70.2
Gloucester	70.3	73.6	65.0
Swindon	92.0	68.0	65.3
Taunton	61.4	75.0	68.9
Truro	84.4	82.2	66.1
	74.6	**76.1**	**66.7**

5.3.3 Prevalence of *ampC*-Carrying Plasmids

All 276 isolates were tested for the presence of CIT and DHA plasmid genes using two separate simplex PCR assays. The isolates allocated to the Non-ESBL group were further tested for the other four AmpC plasmid gene groups using the same two multiplex assays as in the pilot study (CIT/DHA/ACC and FOX/MOX/EBC).

Overall, a total of 19 isolates had a PCR result compatible with the presence of an *ampC*-carrying plasmid. All of these isolates were in the non-ESBL group. Fifteen isolates had a plasmid gene from the CIT group and four isolates were carrying a

plasmid gene from the DHA group. None of the other four AmpC plasmid gene groups were detected in the multiplex screening assays.

The 19 isolates with an *ampC*-carrying plasmid were investigated further to identify the specific plasmid gene involved. Each of the PCR products from the simplex reactions were referred to the external sequencing company, using the relevant forward amplification primer as the sequencing primer. Full sequences were obtained for 14 of the 15 isolates with a CIT-type plasmid gene. When aligned using ClustalW, the sequences showed 100% identity with each other and with a reference sequence for CMY-2 (GenBank Acc. No. AB212086) (**Figure 5-2**).

The consensus sequence obtained from the alignment was analysed using the BLAST (Basic Local Alignment Search Tool) located at http://blast.ncbi.nlm.nih.gov/Blast.cgi. Results indicated similarity with a number of hits, due to the relatively small portion of the gene sequenced. There were a total of 112 registered sequences that gave 100% identity with the query sequence across the 303bp (**Figure 5-3**). These included CMY-2 as a potential match, but also included some other AmpC plasmid genes too. The full hit table is given in the appendices (Section 9.10).

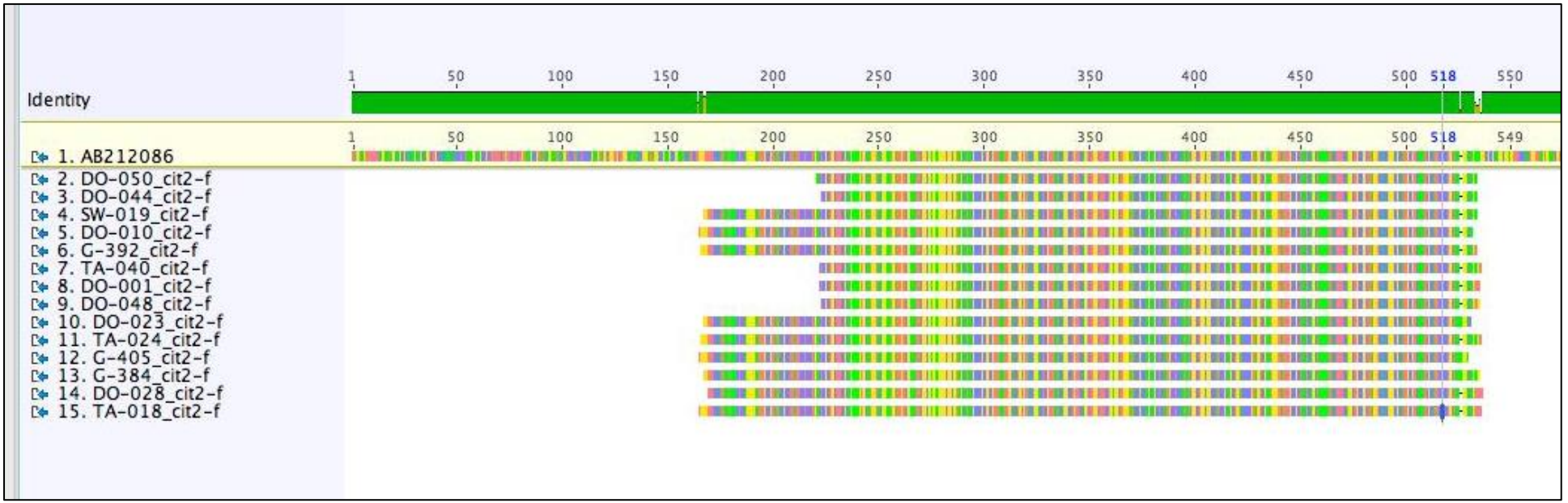


Figure 5-2: Alignment of sequences from 14 isolates with CIT-type plasmid gene.
Alignment was done using the ClustalW plugin module in the Geneious software. The green bar at the top indicates 100% identity across the sequence.

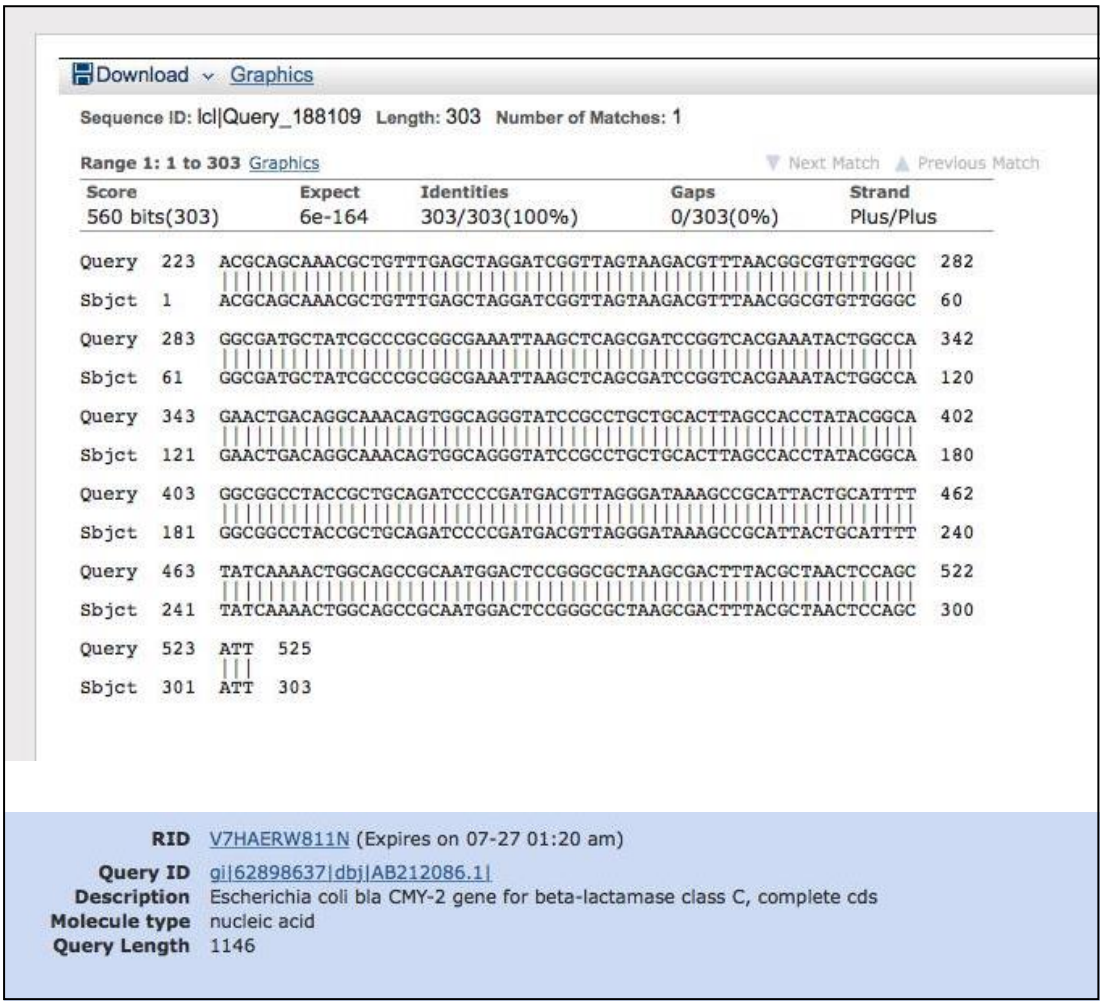
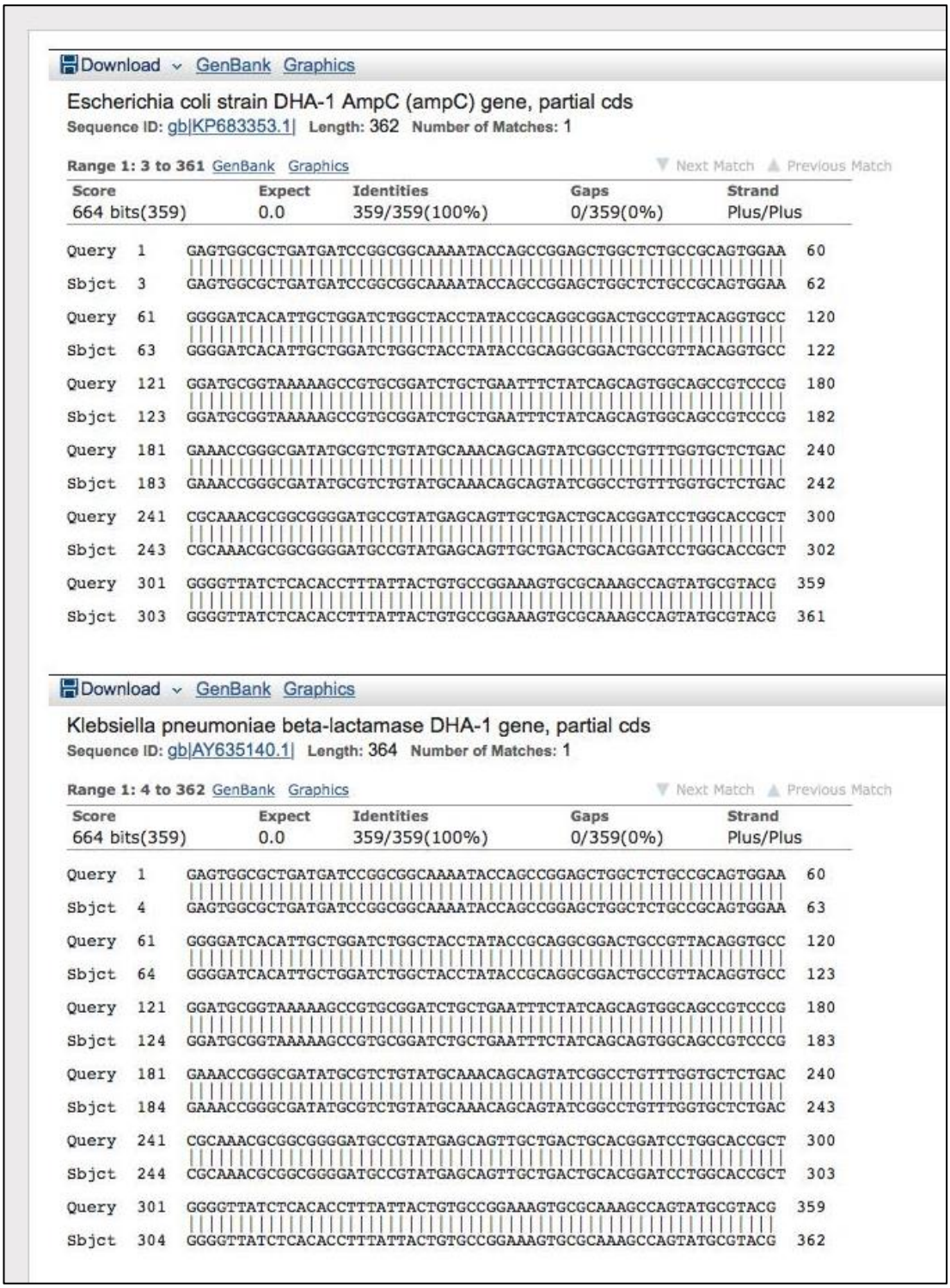


Figure 5-3: Alignment view of the CMY-2 plasmid gene match from the BLAST query.
The consensus sequence extracted from the alignment of the 14 isolates was submitted to the BLAST query software.

The alignment of the four isolates with a DHA-type plasmid gene demonstrated 100% identity with each other. The BLAST results indicated the highest level of similarity with 100 registered GenBank sequences, including those for the DHA-1 gene. The full list is provided in the appendices (Section 9.11). The DHA consensus sequence also showed 100% identity with the GenBank sequences for DHA-1 in *E. coli* and *K. pneumoniae* (Accession Nos. KP683353 and AY635140, respectively) (**Figure 5-4**).



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Escherichia coli strain DHA-1 AmpC (ampC) gene, partial cds

Sequence ID: gb|KP683353.1| Length: 362 Number of Matches: 1

Range 1: 3 to 361 GenBank Graphics Next Match Previous Match

Score	Expect	Identities	Gaps	Strand
664 bits(359)	0.0	359/359(100%)	0/359(0%)	Plus/Plus

```
Query  1    GAGTGGCGCTGATGATCCGGCGGCAAAATACCAGCCGGAGCTGGCTCTGCCGCAGTGGAA  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3    GAGTGGCGCTGATGATCCGGCGGCAAAATACCAGCCGGAGCTGGCTCTGCCGCAGTGGAA  62

Query  61   GGGGATCACATTGCTGGATCTGGCTACCTATACCGCAGGCGGACTGCCGTTACAGGTGCC  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  63   GGGGATCACATTGCTGGATCTGGCTACCTATACCGCAGGCGGACTGCCGTTACAGGTGCC  122

Query  121  GGATGCGGTAAAAAGCCGTGCGGATCTGCTGAATTTCTATCAGCAGTGGCAGCCGTCCCG  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  123  GGATGCGGTAAAAAGCCGTGCGGATCTGCTGAATTTCTATCAGCAGTGGCAGCCGTCCCG  182

Query  181  GAAACCGGGCGATATGCGTCTGTATGCAAACAGCAGTATCGGCCTGTTTGGTGCTCTGAC  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  183  GAAACCGGGCGATATGCGTCTGTATGCAAACAGCAGTATCGGCCTGTTTGGTGCTCTGAC  242

Query  241  CGCAAACGCGGCGGGGATGCCGTATGAGCAGTTGCTGACTGCACGGATCCTGGCACCGCT  300
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Sbjct  243  CGCAAACGCGGCGGGGATGCCGTATGAGCAGTTGCTGACTGCACGGATCCTGGCACCGCT  302

Query  301  GGGGTTATCTCACACCTTTATTACTGTGCCGGAAAGTGCGCAAAGCCAGTATGCGTACG  359
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  303  GGGGTTATCTCACACCTTTATTACTGTGCCGGAAAGTGCGCAAAGCCAGTATGCGTACG  361
```

Download GenBank Graphics

Klebsiella pneumoniae beta-lactamase DHA-1 gene, partial cds

Sequence ID: gb|AY635140.1| Length: 364 Number of Matches: 1

Range 1: 4 to 362 GenBank Graphics Next Match Previous Match

Score	Expect	Identities	Gaps	Strand
664 bits(359)	0.0	359/359(100%)	0/359(0%)	Plus/Plus

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Query  1    GAGTGGCGCTGATGATCCGGCGGCAAAATACCAGCCGGAGCTGGCTCTGCCGCAGTGGAA  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4    GAGTGGCGCTGATGATCCGGCGGCAAAATACCAGCCGGAGCTGGCTCTGCCGCAGTGGAA  63

Query  61   GGGGATCACATTGCTGGATCTGGCTACCTATACCGCAGGCGGACTGCCGTTACAGGTGCC  120
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Sbjct  64   GGGGATCACATTGCTGGATCTGGCTACCTATACCGCAGGCGGACTGCCGTTACAGGTGCC  123

Query  121  GGATGCGGTAAAAAGCCGTGCGGATCTGCTGAATTTCTATCAGCAGTGGCAGCCGTCCCG  180
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Sbjct  124  GGATGCGGTAAAAAGCCGTGCGGATCTGCTGAATTTCTATCAGCAGTGGCAGCCGTCCCG  183

Query  181  GAAACCGGGCGATATGCGTCTGTATGCAAACAGCAGTATCGGCCTGTTTGGTGCTCTGAC  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  184  GAAACCGGGCGATATGCGTCTGTATGCAAACAGCAGTATCGGCCTGTTTGGTGCTCTGAC  243

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Sbjct  244  CGCAAACGCGGCGGGGATGCCGTATGAGCAGTTGCTGACTGCACGGATCCTGGCACCGCT  303

Query  301  GGGGTTATCTCACACCTTTATTACTGTGCCGGAAAGTGCGCAAAGCCAGTATGCGTACG  359
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  304  GGGGTTATCTCACACCTTTATTACTGTGCCGGAAAGTGCGCAAAGCCAGTATGCGTACG  362
```

Figure 5-4: Alignment view of the DHA-1 plasmid gene match from the BLAST query.
The consensus sequence extracted from the alignment of the 4 isolates was submitted to the BLAST query software.

5.3.4 Presence of Mutations in the *ampC* Promoter Region

All 276 isolates were investigated for the presence of mutations in the chromosomal *ampC* promoter region. Amplification failed with primers for two of the isolates (DO-014 and G-375). Two further attempts were made, but without success. The species identification of these two isolates was repeated using an API20E strip. DO-014 was confirmed as being *E. coli*. G-375 was identified as a *Citrobacter freundii* and was excluded from further analysis.

Analysis of the sequences in the remaining 274 isolates revealed mutations at 28 different positions throughout the 271bp region (**Table 5-4**). Geneious software (Version 7.1.7) was used to align all the sequences to the reference sequence (GenBank Accession No: AY899338.1). This was done using the Geneious alignment tool. Mutations and insertions could be easily identified from the alignment output, using an annotated version of the reference sequence (**Figure 5-5**).

Table 5-4: Mutations present in the 271bp amplified region of the *E. coli ampC* promoter gene.
The locations were numbered according to the system described by Jaurin *et al.* (1981). Position +1 is the start position of the transcription process.

Position	Mutation	No. of Isolates	% of Isolates
-82	A to G	26	9.5
-76	G to A	11	4.0
-73	C to T	192	69.8
-42	C to T	9	3.3
-32	T to A	36	13.1
-28	G to A	148	53.8
-20ins	T insertion	2	0.8
-18	G to A	26	9.5
-13ins	G or T insertion	7	2.8
-11	C to T	1	0.4
-1	C to T	26	9.5
+6	C to T	1	0.4
+17	C to T	9	3.3
+21	C to A	1	0.4
+22	C to T	11	4.0
+24	C to A	1	0.4
+26	T to G	11	4.0
+27	A to T	11	4.0
+30	G to A	1	0.4
+31	C to G	1	0.4
+32	G to A	13	4.7
+33	G to A	1	0.4
+34	G to A	5	1.8
+37	G to A, G to T	3	1.1
+58	C to T	60	21.8
+63	T to C	21	7.6
+70	C to T	38	13.8
+81	A to G	82	29.8

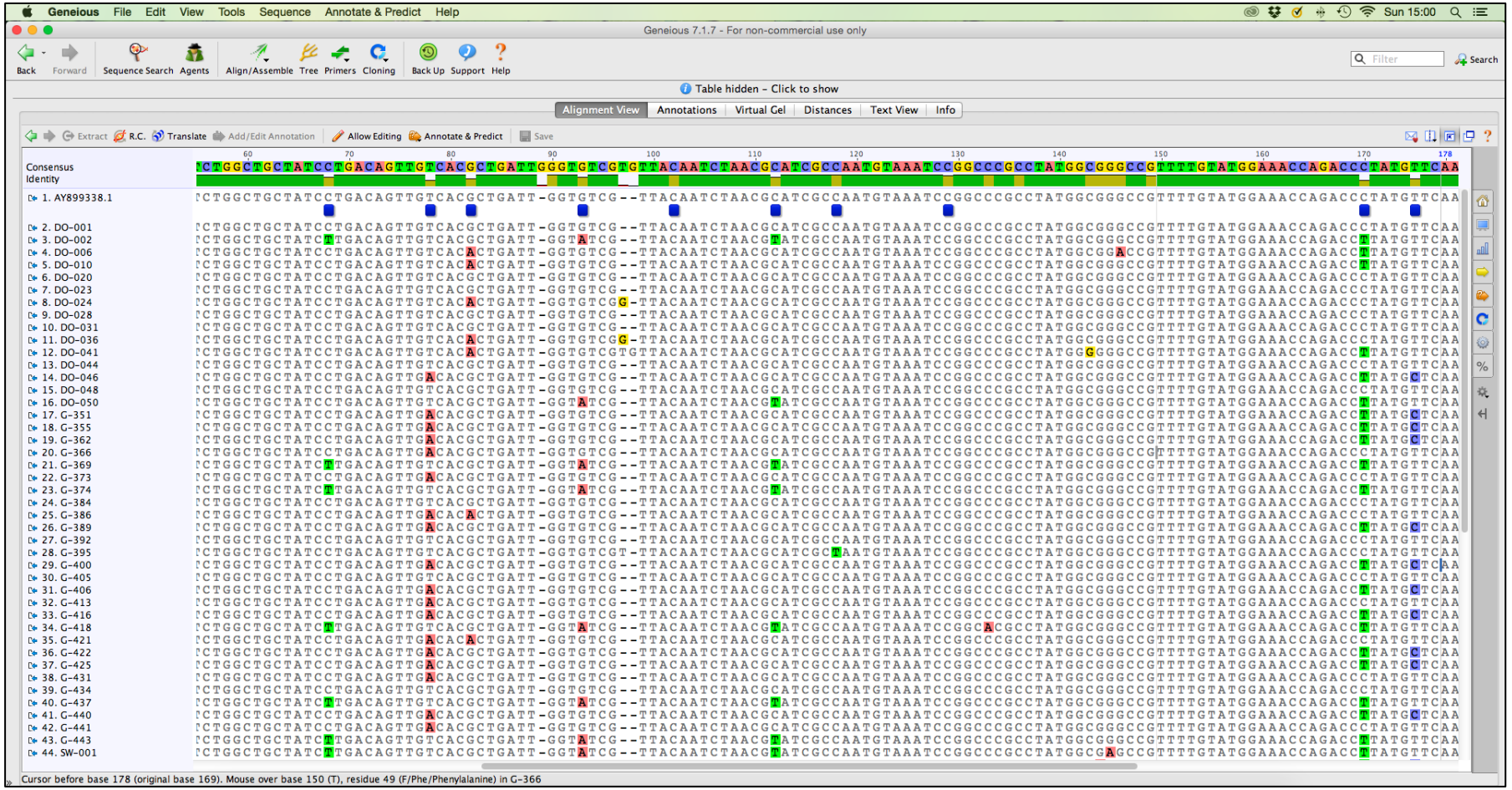

Figure 5-5: Identification of mutations and insertions from the Geneious alignment output, using an annotated version of the reference sequence.
The reference sequence (AY899338 is shown at the top of the alignment, with mutations annotated by blue squares. Differences in nucleotides at each position are highlighted using different colours.

The most common mutation observed was a C to T transition at position -73, which was often seen together with a range of other polymorphisms. This mutation was only seen on its own in 8 isolates (2.2%). The second most common mutation was the -28 transition of G to A, seen in over half of all isolates. This polymorphism was always seen together with mutations at other locations.

The three key mutations seen in the pilot study (-42, -32 and -13ins), and described by Tracz *et al*. (2007), were again observed in the regional study isolates. Nine isolates (3.3%) had the -42 mutation, 13.1% isolates (n=36) had the -32 mutation present and 2.8% of isolates (n=7) had one or two bases inserted at position -13. None of the three key *ampC* promoter mutations were seen in the ESBL group of isolates. Together with the AmpC plasmid genes identified in 19 isolates, the presence of *ampC* chromosomal mutations and acquired *ampC* genes was confirmed in 90.9% of the 76 isolates tested in the Non-ESBL group.

Phylogenetic analysis of the 76 *ampC* promoter sequences (**Figure 5-6**) demonstrates how the isolates with the -42 mutation and those carrying the CIT-type plasmid genes are clustered into separate groups, clearly distinct from the main population of isolates with the -32 mutation. There are five isolates with AmpC plasmid genes that are clustered with the main group. It is possible that these are similar in nature to the main group, but that have acquired the plasmid genes. Studies have indicated that isolates with chromosomal mutations are more likely to be commensal strains (Corvec *et al.*, 2007).

5.3.5 Prevalence of ESBL Resistance Genes in *E. coli*

A total of 151 isolates (54.9%) were positive for the Group 1-specific CTX-M assay. Of the remaining isolates, three subsequently gave PCR reactions indicative of the presence of another type of CTX-M ESBL gene. There were 44 isolates with a positive clavulanic acid synergy result that did not have PCR results indicating the presence of a CTX-M gene. These isolates are likely to have either a SHV or TEM ESBL gene present, but were not investigated further.

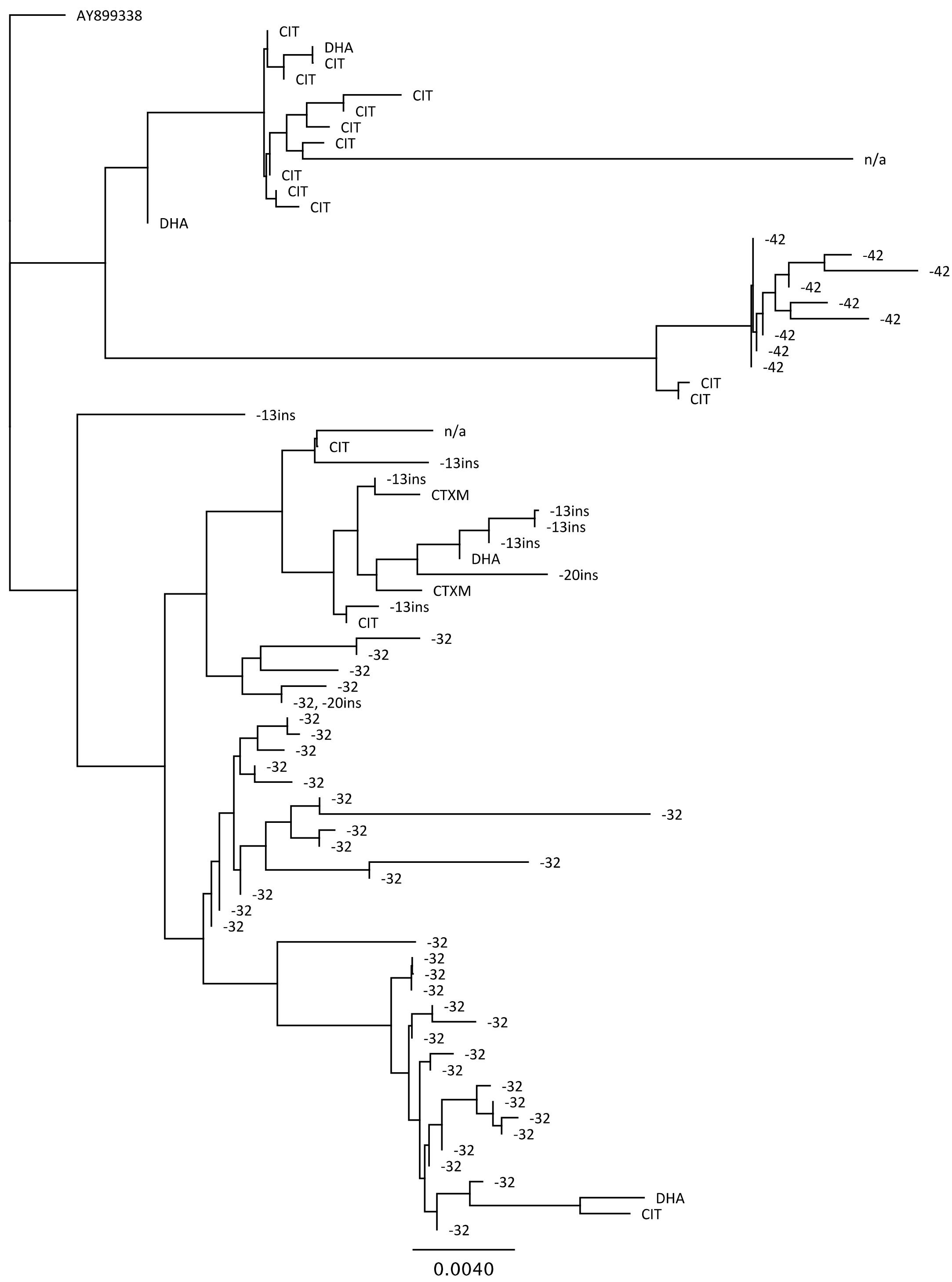

Figure 5-6: Phylogenetic tree of the 76 isolates of *E. coli* in the Non-ESBL group.

Tree was created in the Geneious software package (Version 7.1.7), using the Jukes-Cantor model and Neighbor-Joining method. The labels indicate the primary *ampC* mutation present (-42, -32, -20ins or -13ins), presence of an AmpC plasmid gene (CIT or DHA) or the presence of CTX-M ESBL genes. n/a indicates an unknown mechanism.

There were 4 isolates that were negative with the clavulanic acid synergy test, but which later gave a positive result for a CTX-M Group 1 ESBL in the PCR assay. This highlights one of the limitations of the phenotypic method for detecting ESBL enzymes. In these four cases, the zone sizes for both cefpodoxime and cefpodoxime with clavulanic acid were 0mm, making interpretation impossible. The interpretation of the combination disc method relies on a ≥5mm difference in the two zone sizes to identify a positive result (M'Zali *et al.*, 2000).

5.3.6 Summary of Resistance Mechanisms

Having completed the PCR assays for detecting the various resistance genes, isolates were allocated to one of six groups based on the evidence of the genotypic mechanism present (**Table 5-5**).

Table 5-5: Criteria used for allocating the *E. coli* isolates to one of six groups for the identified resistance mechanism present.

Group	Criteria
CTX-M Type ESBL	Evidence of cephalosporin / clavulanic acid synergy with a positive PCR assay for the universal CTX-M primers (negative for Group 1).
CTX-M-Group 1 ESBL	Evidence of cephalosporin / clavulanic acid synergy with a positive PCR assay for the Group 1 specific CTX-M primers
Non-CTX-M ESBL	Evidence of cephalosporin / clavulanic acid synergy, but with negative PCR assays for the universal and Group 1 specific CTX-M primers
AmpC BL (plasmid-mediated)	No evidence of cephalosporin / clavulanic acid synergy, but with a positive AmpC plasmid gene PCR assay
AmpC BL (chromosomal)	No evidence of cephalosporin / clavulanic acid synergy, but with presence of key chromosomal promoter region mutations (-42, -32 and -13ins)
Other	No evidence of cephalosporin / clavulanic acid synergy, and no evidence of AmpC mechanism present

Although isolates could have been allocated to more than one group, all in fact were assigned to just one group each based on the results of the PCR and phenotypic tests. The proportion of isolates in each group for the laboratories is provided in **Figure 5-7**.

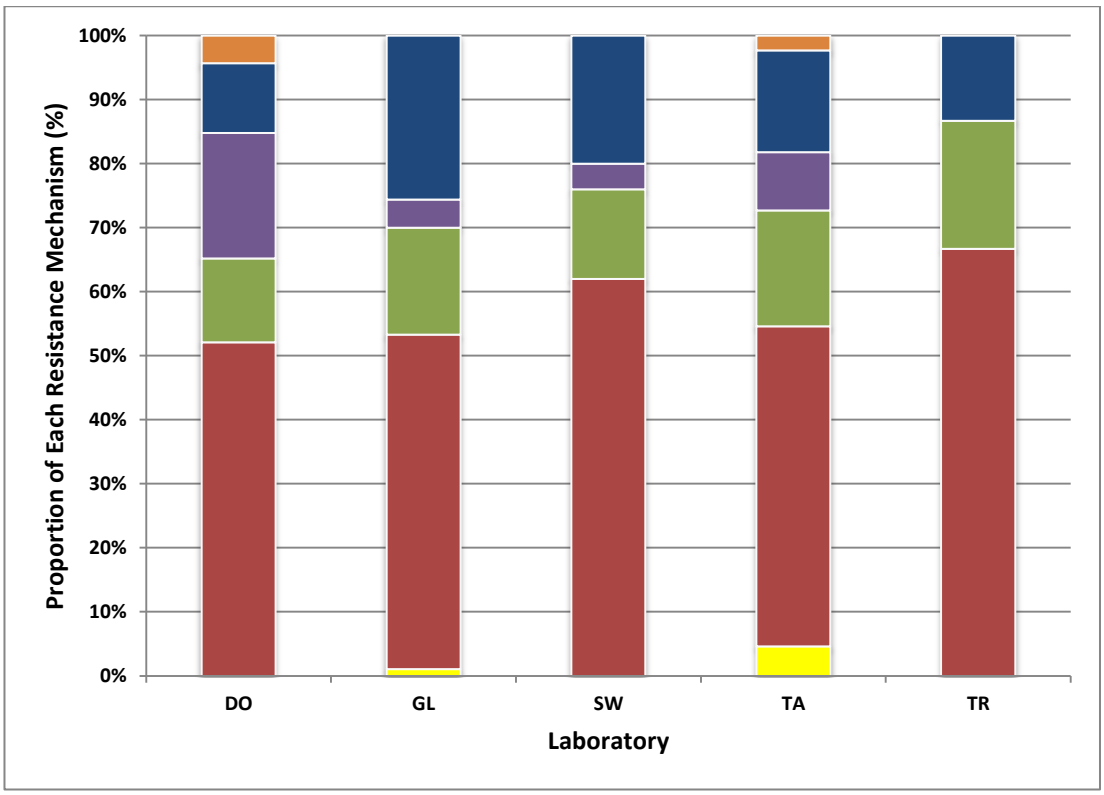

Figure 5-7: Proportions (%) of *E. coli* isolates in each of the resistance mechanism groups.
Lab Key: Dorchester (DO), Gloucester (GL), Swindon (SW), Taunton (TA) and Truro (TR).

Colour Key: CTX-M (Other) – Yellow; CTX-M Group 1 – Red; Non CTX-M – Green; AmpC Plasmid – Purple; AmpC Chromosomal – Blue; Other Mechanism – Orange.

5.3.7 Designation of Phylogroups from *ampC* Promoter Region Mutations

In total, 31 different phylogroups were allocated from the patterns of mutations in the 28 locations in the *ampC* promoter region (**Table 5-6**). There were 21 additional phylogroups recorded compared to the pilot study isolates, and there were 9 phylogroups seen in the pilot study that were not recorded in the regional study (G3, G6, G7, G10, G11, G12, G14, G15 and G16). There were significant differences in the phylogroups observed between the ESBL and Non-ESBL groups of isolates (**Figure 5-8**).

Table 5-6: Details of the 31 AmpC phylogroups identified from sequencing *E. coli* isolates collected in the regional study.

The bold underlined characters indicate the key mutations responsible for AmpC enzyme hyper-production, as described by Tracz *et al.* (2007).

*Phylogroups G1 to G18 were also detected in the pilot study stage.

(Numbers G3, G6, G7, G10, G11, G12, G14, G15 and G16 were allocated in the pilot study, but were not observed in isolates from the regional study).

WT indicates a wild type isolate, with no identified mutations.

Type	Mutations	No. of Isolates	% of Isolates
WT	none	1	0.4
G0	+81	13	4.7
G1*	-73, **-32**, +58, +63	16	5.8
G2*	**-32**	11	4.0
G4*	-73, **-13ins**, +6	1	0.4
G5*	-82, **-42**, -18, -1, +58, +81	7	2.5
G8*	-82, -18, -1, +58, +81	17	6.2
G9*	-73, -28	119	43.3
G13*	-73, **-32**, -28	3	1.1
G17*	-73, -28, +34, +58	4	1.5
G18*	-73, -28, +17	5	1.8
G19	+70, +81	31	11.3
G20	-73, +58, +63	4	1.5
G21	-73, -28, +58	7	2.5
G22	-73, -28, **-13ins**	2	0.7
G23	-73, -28, **-13ins**, +31, +58	1	0.4
G24	-82, **-42**, -18, -1, +33, +58, +81	1	0.4
G25	-73, +32, +58, +63	1	0.4
G26	-73, **-32**, -28, -20ins	1	0.4
G27	-73, **-32**, +28, +37	1	0.4
G28	-73, -28, **-13ins**, +17	2	0.7
G29	-73, **-32**, -28, +58	1	0.4
G30	-73, -28, **-13ins**, +17	1	0.4
G31	-73, **-32**, -28, -11	1	0.4
G32	-73, -28, -20ins, +17, +34	1	0.4
G33	-73, **-32**, +32, +37	1	0.4
G34	-82, **-42**, -18, -1, +21, +58, +81	1	0.4
G35	-73, **-32**, +37	1	0.4
G36	-73	8	2.9
G37	-76, +22, +26, +27, +32, +70, +81	6	2.2
G38	-76, +22, +26, +27, +32, +81	5	1.8
G39	+30, +70, +81	1	0.4

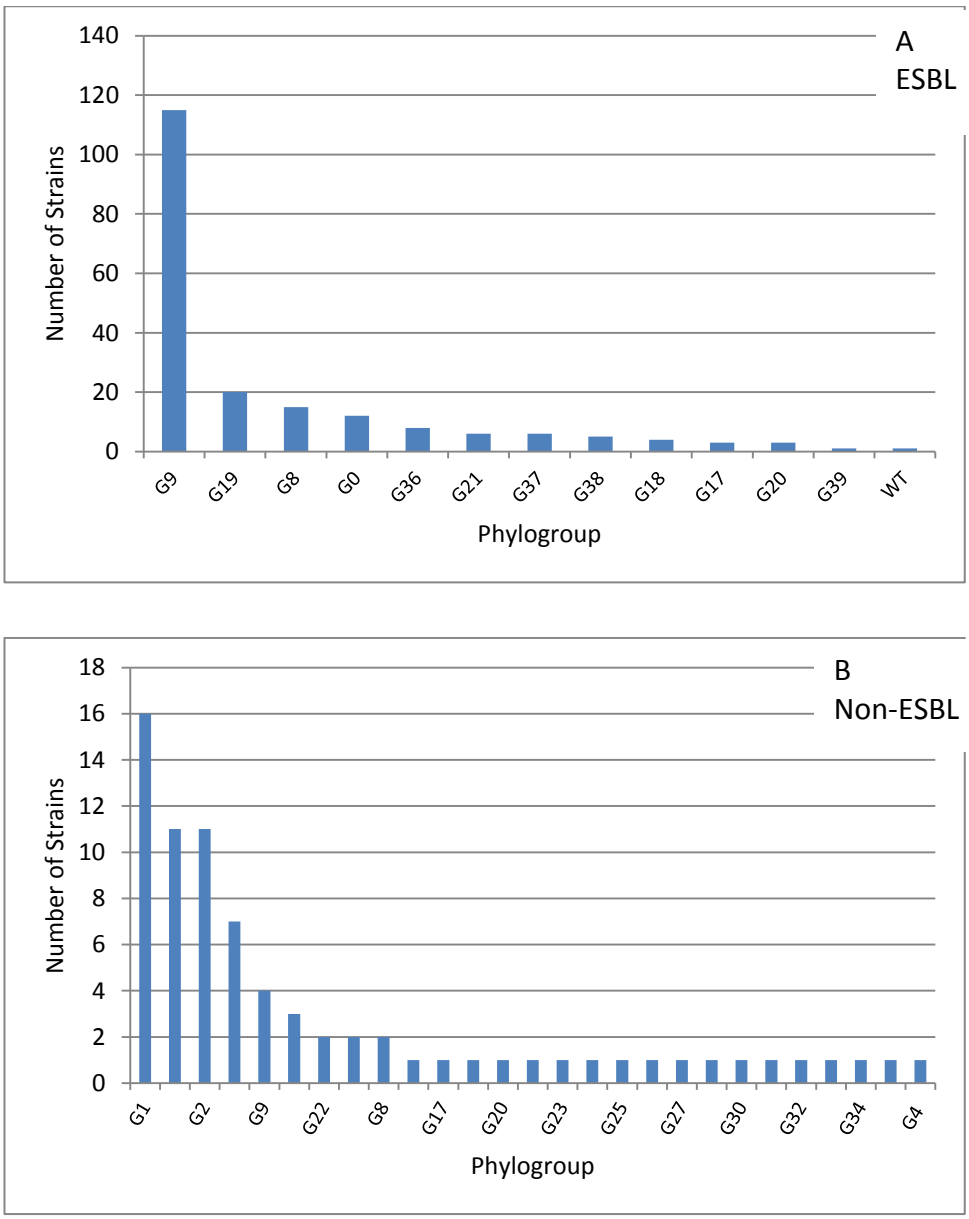

Figure 5-8: Number of *E. coli* isolates recorded in each of the AmpC phylogroups.
Numbers are indicated on each graph for A) the ESBL Group comprising 199 isolates and 13 phylogroups and B) the Non-ESBL group comprising 76 isolates and 27 phylogroups.
WT indicates a wild type isolate, with no identified mutations.

The most common phylogroup overall was G9, which was observed in 43.3% of isolates (n=119). This phylogroup had mutations at positions -73 and -28 and was seen mainly in isolates in the ESBL group. G19 was the second most common phylogroup, seen in 11.3% (n=31) of all isolates. Although 20 of the G19 isolates were again from the ESBL group, there were 11 isolates from the Non-ESBL group; all of which were identified as carrying an AmpC plasmid gene. As was seen in the pilot study, the most common phylogroup in the Non-ESBL group was G1; represented by 21.1% of the 76 isolates and including the key chromosomal mutation at position -32. Overall therefore, >50% of all isolates were allocated to one of just two phylogroup patterns (G9 and G19). The variations in phylogroups detected in each of the different laboratories in shown in **Table 5-7**.

Table 5-7: Analysis of different phylogroup patterns for the five laboratories in the regional study.
The number of each phylogroup observed in isolates collected from each laboratory is given. Phylogroup patterns are listed in order of frequency of detection.
Lab Key: Dorchester (DO), Gloucester (GL), Swindon (SW), Taunton (TA) and Truro (TR).

Genotype	DO	GL	SW	TA	TR	Total
G9	13	29	23	22	29	119
G19	12	12	4	3		31
G8	2	6	3	3	3	17
G1	1	10	2	2	1	16
G0	3	6	3		1	13
G2		5	2	2	2	11
G36	2	1		4	1	8
G5	1	4		1	1	7
G21	2	4			1	7
G37	2	2	1		1	6
G18	1	2		1	1	5
G38	1	2	1	1		5
G17	1	3				4
G20	1		3			4
G13		2	1			3
G22	2					2
G28			1		1	2
G30				1		1
G27			1			1
G39				1		1
G31				1		1
G29			1			1
G32					1	1
G26			1			1
G33					1	1
G4		1				1
G34		1				1
G23	1					1
G35				1		1
WT					1	1
G24			1			1
G25			1			1
Total	**45**	**91**	**50**	**44**	**45**	**275**

5.4 Discussion

5.4.1 Patient Demographics

In this regional study, 74.6% of samples received were from female patients and the mean age was 66.7 years (range of 1 to 102 years). This compares with the smaller pilot study of the 50 isolates collected at the Gloucester laboratory (Lewis *et al.*, 2015), in which 75% of samples were from females and the mean age was 58.3 years.

Only six of the isolates in the current study originated from blood culture samples. Although laboratories were asked to submit isolates from all sample types, the population of isolates was heavily biased towards the urine samples. It was not possible, therefore, to investigate whether there were differences between blood and urine samples as two groups. It is more likely for isolates to have been collected from urine and blood culture samples, due to the routine identification of *E. coli* to species level in these sample types. Isolates of *E. coli* from other sample types are commonly reported in clinical laboratories as a generic "coliform" identification.

Although the current study collected more isolates from urine samples than any other sample type, this still represents an important population. Not only is there a considerable amount of morbidity associated with urinary tract infections, but these infections are known to act as a primary source for more serious infections such as bacteraemia (Horner *et al.*, 2013). Davies *et al*. (2012) reported that 45% of bacteraemia cases caused by *E. coli* had a urinary tract infection as the primary source.

5.4.2 Epidemiology of ESBL-Mediated Resistance

The presence of bla_{ESBL} genes was not investigated as part of the pilot study, as the focus was mainly on *ampC* genes. In the regional study, however, there was phenotypic evidence of ESBL-mediated resistance in 71.4% of the isolates collected (**Table 5-2**). All the isolates in this part of the study were included on the basis of identification as *E. coli* and having resistance to cefpodoxime.

Using the PCR assays developed in Chapter 3, the presence of CTX-M Group 1 ESBL genes was confirmed in 54.9% of the isolates overall, and in 76.6% of the isolates with an ESBL phenotype. This confirms the importance of these genes as a cause of cephalosporin resistance in *E. coli*. Although the identification of the actual CTX-M gene present was not completed, it is likely that the majority of these were CTX-M-15.

Woodford *et al*. (2004) tested 287 *E. coli* isolates with a CTX-M phenotype (defined as having a cefotaxime MIC of ≥8-fold higher than ceftazidime, both reduced in the presence of clavulanic acid). A total of 96% of the isolates were PCR positive for CTX-M Group 1 genes (includes CTX-M-15). Hopkins *et al*. (2006a) found 31 isolates with a CTX-M gene present in an early collection of isolates submitted to the UK national reference laboratory. Of these, 29 isolates were confirmed as having CTX-M-15 (Group 1) genes. Enoch *et al*. (2012) investigated 67 isolates in the UK and found that 58% were positive for CTX-M-Group 1 genes, 18% had CTX-M-Group 9 genes, one isolate had both and 22% had neither present. Horner *et al*. (2013) found 68% of 63 ESBL-positive *E. coli* isolates were identified as CTX-M Group 1.

Only a small number of isolates (n=3) were positive for the universal CTX-M primers, but negative for the Group 1-specific primers. This was somewhat surprising, as a higher number of other $bla_{\text{CTX-M}}$ genes had been expected to be present. This is particularly the case for CTX-M genes in Group 9, such as CTX-M-9 and CTX-M-14. Courpon-Claudinon *et al*. (2010) investigated 1081 bacteraemia *E. coli* isolates in France, finding 18 with CTX-M genes; identified as CTX-M-15 (n=9), CTX-M-14 (n=6), CTX-M-1 (n=2) and CTX-M-9 (n=1). Wickramasinghe *et al*. (2012) investigated the carriage of ESBL-producing strains in faecal samples submitted for routine microbiological analysis. Of the 80 ESBL-producing *E. coli* isolates found, 72.5% were CTX-M-15 and 16.3% were CTX-M-9, with one isolate having both genes present.

The presence of other ESBL genes (SHV and TEM) was not investigated as part of this study. However, it is likely that the remaining 43 isolates with an ESBL phenotype were producing one of these types of ESBL enzymes. Voets *et al*. (2012)

found that 14.4% of 479 *E. coli* isolates had one of either a SHV or TEM type ESBL present. Izdebski *et al*. (2013) reported proportions of bla_{SHV} and bla_{TEM} genes of 13.5% and 1.1% in 376 *E. coli* isolates, respectively.

5.4.3 Association of ESBL Type with Location

In the regional study in London and the South East, Potz *et al*. (2006) found the prevalence of CTX-M producing isolates varied between 12 laboratories, from 0.6% to 4.3%. There was a similar variation observed when all ESBL types were included. These are comparable with the data from an informal audit conducted in 2013 (Lewis, 2013), in which the prevalence of ESBL-producing isolates found in urine samples varied between 1.3% and 6.2% for five laboratories in the South West region (data provided in the Appendices, Section 9.12).

In the current study, the denominator data for laboratory isolation rates were not collected; so overall prevalence rates are not available. However, the proportions of cephalosporin-resistant *E. coli* isolates with confirmed ESBL resistance (genotypic and phenotypic) did vary significantly between laboratories. The Dorchester and Truro laboratories had the biggest differences in rates of ESBL genes present, at 65.2% for Dorchester and 86.7% for Truro (p=0.02, Fisher's Exact test). This compared to 30.4% and 13.3%, respectively for the confirmed AmpC-producing isolates (not quite significant at p=0.08). Variations in resistance rates could be indirectly explained by differences in antibiotic prescribing rates. Nearly 80% of all antibiotic prescribing happens in the primary care setting, of which over half is for respiratory tract infections. National data from the NHS Atlas of Variation (Public Health England, 2015a) suggests a four-fold variation in the prescribing rates for key antibiotics (includes cephalosporins) for the different clinical commissioning groups (CCG) in England. Hawker *et al*. (2014) demonstrated that prescribing rates in English GP surgeries for coughs and colds varied between one-third of all presenting patients to two-thirds. Although this does not relate directly to prescribing for urinary tract infections, it is possible that a similar level of variation would exist.

Not all studies, however, have demonstrated evidence of a variation in resistance rates. Horner *et al*. (2013) found no significant variation in the distribution of ESBL

genes between the laboratories in their study of bacteraemia isolates from 14 laboratories in the Yorkshire region.

5.4.4 Epidemiology of AmpC-Mediated Resistance

A total of 19 of the 276 isolates (6.9%) carried an AmpC plasmid gene; 15 with a CIT-type and 4 with a DHA-type. These isolates were all in the Non-ESBL group. Sequencing the PCR products with the forward amplification primer provided mixed results in trying to identify the actual plasmid gene present. Other studies have used different primer sets to ensure a larger portion of the plasmid gene is amplified. In the original AmpC plasmid paper, for example, the authors used different primers to sequence the whole region flanking the CMY-2 gene (Perez-Perez & Hanson, 2002).

Although likely to be CMY-2 plasmid genes, the CIT type products detected by PCR in this study may actually be caused by other plasmid-mediated genes. Based on evidence from other studies, one cannot rule out the possibility of a different or novel plasmid gene being present. Hopkins *et al*. (2006b) discovered a new CIT-type plasmid gene (CMY-21) which shared 99% identity with the known CMY-2 gene.

In the current study, results of the sequencing of the chromosomal *ampC* promoter region were available for 274 of the isolates (**Table 5-4**). The sequencing analysis revealed mutations at 28 different positions in the amplified region, compared to just the 16 positions found in the pilot study. There were a total of 31 different phylogroups detected, compared to the 18 allocated in the pilot study.

Within the ESBL group, there were 12 different phylogroups present. The most commonly observed mutations in this group were at position -73 (70.1%) and position -28 (64.5%). The most common phylogroup in the ESBL group was G9, which included both the -73 and -28 mutations. The -28 mutation was always seen together with the -73, but not vice versa. There were also mutations in the coding region at positions +70 and +81 in 13.2% and 29.4% of isolates, respectively. The -18 mutation was seen in 15 of the isolates in the ESBL group. Observed in the pilot study together with the -32 and -13ins mutations, the -18 and -28 mutations are

thought to have a minimal impact on the level of enzyme production (Tracz *et al.*, 2007). Corvec *et al*. (2007) also demonstrated that the -18 and -28 mutations are present in *E. coli* isolates that are fully sensitive.

Within the AmpC group, the three key mutations identified in the pilot study (-42, -32 and -13ins) were observed in a total of 51 isolates (66.2%). Again, the most common of these mutations was the T to A transition at position -32, seen in a total of 36 isolates. The -42 and -13ins mutations were seen in 9 isolates and 6 isolates, respectively.

A similar range of mutation patterns were seen, when compared with the results of the pilot study. Mutations in the wild-type promoter were observed primarily at position -32. The creation of a displaced promoter was seen with mutations at positions -42 and -18 and six of the isolates had an increased spacer region from insertions at position -13.

There were two isolates with insertions at position -20 (SW-007 and TR-032) (**Figure 5-9**). Not seen in the pilot study or the large Canadian study (Mulvey *et al.*, 2005), this may be the first report of insertions at this location in the UK. In both cases, the insertion was a thymine base, and was always seen in association with -73 and -28 mutations. One isolate had a -32 mutation as well. Both isolates originated from different laboratories (Swindon and Truro), suggesting that this type of mutation may be reasonably widespread throughout the region. Jorgensen *et al*. (2010) also reported one isolate with a thymine base insertion at this position. A recent study in Spain identified two isolates with a mutation at position -20, and confirmed a corresponding increase in enzyme production of 20-fold (Alonso *et al.*, 2016).

Within the whole group of isolates, there were a total of 15 of the 32 phylogroups that were seen only in one of the laboratories (**Table 5-7**). There were only three phylogroups (G1, G8 and G9) that were seen in all the five laboratories. These three phylogroups were associated primarily with the Non-ESBL group for phylogroup G1 and the ESBL group for phylogroups G8 and G9. This suggests a wide diversity of strains within the region, and in particular for the isolates in the Non-ESBL group. Of

the 17 phylogroups with one of the key *ampC* mutations present (-42, -32, -13ins), 15 were seen in only one or two laboratories.

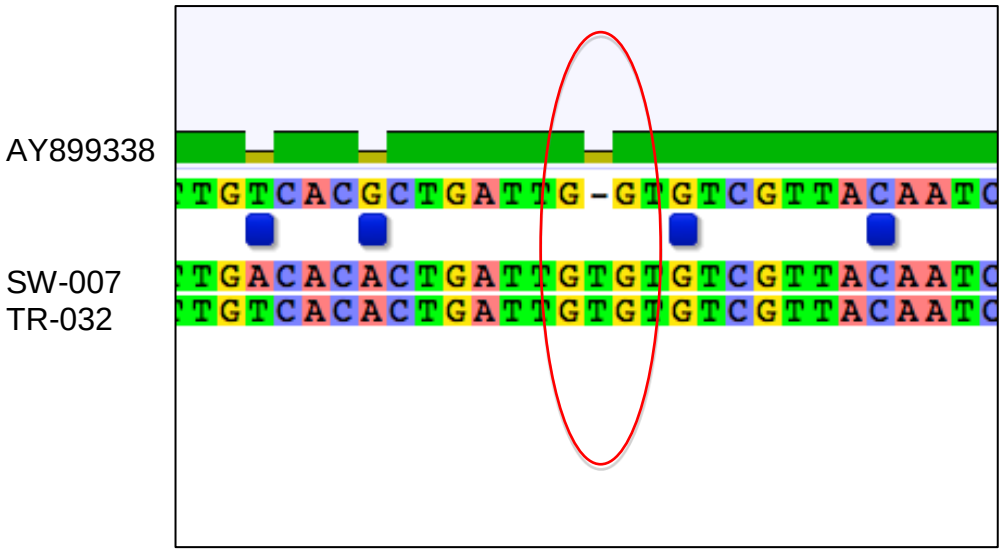

Figure 5-9: Presence of thymine nucleotide insertion at position -20 in two isolates of *E. coli*, increasing the *ampC* promoter spacer region by one base pair.
The two isolates (SW-007 and TR-032) were isolated at different laboratories (Swindon & Truro) in the region.

5.4.5 Association of AmpC Type with Location

The variation in AmpC plasmid gene carriage rates was considerable between the five laboratories (**Table 5-7**), with a range from 0% to 19.6% as a proportion of all isolates. This was found to be a significant difference between the Truro and Dorchester laboratories ($p<0.005$, Fisher's Exact test). To date, no other study has detected such a significant variation between different laboratories.

When looking at the group of isolates most comparable with the pilot study (Gloucester isolates of cefpodoxime-resistant, ESBL-negative *E. coli*), the 27 isolates had *ampC* chromosomal mutations at a total of 15 locations in the amplified sequence. Compared to the 16 locations seen in the pilot study, this suggests that the number and nature of the *ampC* mutations also vary by laboratory location, as the additional mutations seen in the regional study must have come from other locations. This is further supported by observations that the allocated phylogroups

had some variation between laboratories, with a total of 46.9% of the phylogroups being individually unique to their respective locations.

The question of whether the -32 mutation was restricted to, or had a higher prevalence in Gloucester compared to the other laboratories was investigated. The proportion of -32 mutations as a total of potential AmpC isolates (i.e. the Non-ESBL group) showed significant variation between the five laboratories. The lowest prevalence was seen in the Dorchester laboratory at 5.9% (1 of 17 isolates) and the highest was in Gloucester at 63.0% (17 of 27 isolates). However, the -32 mutation was also the most common mutation at the other three laboratories as well: 61.5% in Swindon, 57.2% in Truro and 46.2% in Taunton. This confirms that the higher prevalence of this mutation in AmpC-producing isolates is not unique to the Gloucester laboratory, and is certainly a picture seen throughout most of the South West region. There are, as yet, no other studies published for UK data to confirm whether this holds true for the rest of the country.

5.5 Summary

The data presented in this chapter represent a unique snapshot picture of the cefpodoxime-resistant *E. coli* population in the South West region in 2013. There are relatively few published reports of ESBL and AmpC-mediated resistance in UK strains of *E. coli*, and fewer still that examine patterns over a regional distance. To date, there have been no studies published related to the investigation of strains isolated in laboratories in the South West region.

The isolates were collected over a relatively short period (3 months), so may have been influenced by some collection bias. The differences in AmpC plasmid gene rates between the laboratories may have been due to a short-term increase in prevalence of a particular mechanism. As the collection numbers were limited to 50 isolates for most laboratories, a higher rate of ESBL isolates would reduce the numbers of potential AmpC isolates, and vice versa. A further collection of isolates after a 1-2 year period would have helped to confirm unusual observations, and would also have highlighted trends in resistance or isolation rates.

This study represents the first regional investigation of chromosomal *ampC* mutations in the UK. Similar types and locations of mutations were seen here as have been reported in publications elsewhere. The -32 mutation was the most common of the key mutations (-42, -32 and -13ins) in the pilot study, and this observation was confirmed in the regional study for four of the five laboratories. This has not been the case in any other published study. However, the full implications of a different picture for the UK are uncertain.

This study is also the first in the UK to report the insertion of a single nucleotide at position -20. This mutation was seen in two isolates isolated from different laboratories, so is not restricted to one particular location. The -20 insertion would result in an increased spacer region, similar to the -13 insertion. Tracz *et al*. (2007) reported a 24- to 61-fold increase in enzyme production resulting from a single base pair insertion at position -13. It is not clear at this stage though, whether a similar effect on enzyme production would be observed for the -20 insertion. Further investigations on a larger number of isolates are required.

5.6 Future Work

The majority of isolates collected in this study were from urine samples. Although this represents an important population, a higher number of isolates from blood culture samples would have enabled useful comparisons with other studies. A further study of either local or regional bacteraemia *E. coli* isolates would provide more detailed data on the proportions and types of resistance mechanisms present.

A decision was made at the start of the study to collect all cefpodoxime-resistant isolates for the regional study. Although the pilot study had focussed only on those isolates likely to be producing AmpC enzymes, there was a potential risk that isolates with new or multiple genes present could be missed if the same selection criteria were used for the regional study. The disadvantage of this approach, however, is that a significantly smaller number of AmpC-producing isolates were included in the study. Further collection of cefpodoxime-resistant, ESBL-negative isolates only would help to confirm the significance of the -20 mutation found in this study.

6 Clonal Structure of Regional *E. coli* Isolates

6.1 Introduction

Multi-locus sequence typing (MLST) is a method designed to study the genetic relatedness of a collection of isolates. Its principle was first described in 1998 by Maiden *et al*. (1998), when the DNA sequences of six housekeeping genes were used to type isolates of *Neisseria meningitidis*. The main advantage of the method described at the time was that data would be comparable between laboratories, and would thus enable the creation of global databases to facilitate wider surveillance of strains. Strains are grouped into clonal complexes, based on sharing the same allele sequences at a number of the different loci. Clonal complexes are usually comprised of a single dominant sequence type, with a number of less common close relatives. The emergence of clonal complexes within a population occurs as a particular sequence type (ST) increases in frequency, and as a result of genetic changes over time becomes the dominant founder of the complex (Feil *et al.*, 2004).

Tartof *et al*. (2005) published one of the initial papers on the use of MLST to investigate uropathogenic *E. coli* strains in the United States. A total of 45 *E. coli* isolates from a variety of sources and sample types were tested, giving 17 different sequence types. The majority of the isolates were allocated to the STC69 clonal complex, with ST69 the most common sequence type overall.

In terms of early UK studies, Lau *et al.* (2008) used MLST to investigate cephalosporin-resistant *E. coli* isolates. A total of 88 isolates isolated in North West England from urine samples (68 isolates) and blood cultures (20 isolates) gave 22 different sequence types in 10 clonal complexes. The most common sequence type was the ST131 clone, accounting for 59% of all isolates. The isolates included in the study were described as cephalosporin-resistant, but were not characterised further to determine the actual mechanisms of resistance. Later, in another UK study, Gibreel *et al*. (2012) investigated 300 *E. coli* isolates from urine samples. A total of 102 sequence types were found; the most common being ST73 (17%), ST131 (13%) and ST69 (9%). Half of the isolates were included in just six sequence types.

In one of the few studies to use MLST to investigate potential AmpC-producing *E. coli* isolates, Corvec *et al*. (2010) tested 41 isolates from a range of different clinical samples in France. All the isolates were potential AmpC producers; selected as cefoxitin-resistant and ESBL-negative. Of the 41 isolates, 18 had *ampC* chromosomal mutations (all with the -42 mutation) and 23 carried a CIT-type plasmid gene. The 23 isolates with a CIT-type plasmid gene present were typed using MLST, and the most common sequence types were ST156 (34.8%), followed by ST46 (21.7%), ST354 (8%) and then 8 other individual STs. The authors did not report whether the typing was extended to include the 18 isolates with *ampC* chromosomal mutations.

One of only two sets of published MLST data for isolates with identified *ampC* chromosomal mutations was reported in 2009 (Guillouzouic *et al.*, 2009). The number of isolates included in the French study was quite small. Only 12 clinical samples were included, which were compared against 12 animal isolates and 10 fully sensitive human isolates. The majority of the 12 resistant human isolates had the -42 mutation (n=8), with two having the -32 mutation and two having a -11 mutation. Interestingly, all of the AmpC-producing isolates were allocated to a single complex STC23 (ST88, n=8 and ST23, n=4).

In the current study, MLST was used to type all the isolates collected from the regional laboratories. The Achtman scheme was chosen for this project, as it has a larger number of sequence types and registered isolates in the database; 5216 STs compared to the 687 STs in the Pasteur database (last checked 27th August 2015). Although the Pasteur scheme (Jaureguy *et al.*, 2008) uses 8 genes, it has less sequence types recorded.

The aim of the study was to establish whether dominant clones of *E. coli* were circulating within the population of the South West region. A further aim was to establish whether particular clones were associated with different mechanisms of ESBL and AmpC-mediated resistance.

6.2 Methods

The full details of the MLST methods used are provided in Section 2.7. Using the method described by Wirth *et al*. (2006), fragments of seven housekeeping genes were amplified and sequenced. DNA was extracted from each of the isolates using the mechanical lysis method described in Section 2.3.1. Each of the seven genes was amplified using the respective primers, using the standard SYBR Green PCR method used throughout the project. The primers used were those published on the central database website:

http://mlst.warwick.ac.uk/mlst/dbs/Ecoli/documents/primersColi.

The PCR products were sent to an external company (Eurofins Genomics, Germany) for purification and sequencing. The forward amplification primer was used primarily as the sequencing primer. The reported sequence of each allele was entered onto an online database (http://pubmlst.org/) to obtain the allele profile number (Jolley & Maiden, 2010). Once the full set of seven alleles had been identified, the final sequence type was obtained from entering the profile number into the same database.

The web-based eBURST software (http://eburst.mlst.net/v3/) was used to cluster the MLST types into related clonal groups (Feil *et al.*, 2004). Clonal complexes were identified from groups of sequence types that have MLST genes that are identical at six loci. The diversity of a population was calculated from the sequence type data using the Simpson's Index of Diversity formulae (Hunter & Gaston, 1988), with calculated 95% confidence intervals (Grundmann, Hori & Tanner, 2001).

6.3 Results

6.3.1 Prevalence of Sequence Types

A total of 275 isolates (97.5%) gave complete allele profiles for all seven genes sequenced, resulting in the generation of a sequence type. There were 74 different sequence types assigned from the 275 isolates. ST131 was the most common type, with 120 (43.6%) of the isolates; followed by ST73 (6.2%), ST12 (4.7%) and ST38

(4%). Overall, 68.4% of all isolates were included in just 10 sequence types (**Table 6-1**).

The distribution of sequence types between locations is also provided in **Table 6-1**. ST131 was found in all five locations, but the proportion of isolates varied between the five laboratories, ranging from 30.4% for Dorchester to 64.4% for Truro (**Table 6-2**). The proportion of ST131 isolates for the Gloucester and Truro laboratories was found to be significantly different from the average of the whole population ($p<0.05$, χ^2 test).

The second-most common type (ST73) was found in all laboratories, with the exception of Dorchester. The third-most common type (ST12) was found in just three of the five locations (Gloucester, Swindon and Taunton).

Table 6-1: Distribution of *E. coli* sequence types (ST) by location for the five laboratories in the regional study.
There were an additional 59 types that were present in only one location. Lab Key: Dorchester (DO), Gloucester (GL), Swindon (SW), Taunton (TA) and Truro (TR).

ST	DO	GL	SW	TA	TR	Total
131	14	30	24	23	29	120
73	0	7	3	4	3	17
12	0	9	2	2	0	13
38	4	4	2	1	0	11
69	4	3	2	0	0	9
648	2	4	1	0	1	8
405	2	2	1	1	1	7
88	1	2	0	1	1	5
778	2	2	0	0	0	4
10	0	1	1	0	0	2
23	0	1	0	0	1	2
40	0	1	0	0	1	2
58	0	2	0	0	0	2
95	0	0	0	1	1	2
117	1	0	0	1	0	2
127	0	0	1	0	1	2
141	0	0	2	0	0	2
357	0	0	1	1	0	2
393	1	1	0	0	0	2
617	0	2	0	0	0	2
636	1	0	0	1	0	2

Table 6-2: Comparison of ST131 isolation rates for each laboratory in the regional study.
The comparison of the proportion of ST131 isolates for each laboratory when compared to the average rate for the whole population is indicated by the p-value, as determined by chi-square tests.

Location	Total Isolates Tested	% of ST131 Isolates	P-value
Dorchester	46	30.4	0.072
Gloucester	90	33.3	0.049
Swindon	50	48.0	0.530
Taunton	44	52.3	0.246
Truro	45	64.4	0.005
Total	**275**	**43.6**	

6.3.2 Association of Sequence Type with Genotypic Mechanism Present

The association of resistance mechanism with sequence type is provided in **Table 6-3**. The most common sequence type (ST131) was predominantly associated with the CTX-M and ESBL isolates. A total of 94.2% of the isolates with an ST131 profile were either confirmed as having *bla*$_{CTX-M}$ genes by PCR, or were in the phenotypic ESBL group. There was one ST131 isolate (TA-024) with an AmpC plasmid gene present (CIT-type), and 6 isolates that had a chromosomal *ampC* mutation (3 with the -32 mutation and 3 with insertions at position -13).

The second-most common sequence type (ST73) was predominantly associated with the chromosomal *ampC* genotype. A total of 88.2% of these isolates had one of the key *ampC* mutations, nearly all of which were the -32 mutation. The ST12 sequence type was the third most common, and was again associated primarily with the chromosomal *ampC* genotype. A total of 92.3% of these isolates had the -32 mutation present, with the one other ST12 isolate carrying an AmpC plasmid gene.

In general, the sequence types were associated with either ESBL or AmpC mechanisms, or in some cases with ESBL mechanisms and AmpC plasmid genes. Sequence types ST38 and ST69 were predominantly associated with ESBL-producing isolates, but did have two isolates with AmpC plasmid genes present. ST648 had 3 isolates with CTX-M genes present and 3 isolates with an AmpC plasmid gene

present. ST778 was exclusively associated with isolates carrying AmpC plasmid genes, all of which were carrying the CIT-type.

Table 6-3: Distribution of *E. coli* sequence types (ST) by different resistance mechanisms.
There were an additional 59 types that were present only in one mechanism group. CTX-M isolates were identified using the universal or Group 1 specific PCR assay. ESBL isolates were non-CTX-M isolates with phenotypic evidence of ESBL production. AmpC plasmid isolates were confirmed by PCR to be carrying either a CIT- or DHA-type plasmid gene. The AmpC chromosomal isolates were identified as having one of the key mutations present in the *ampC* promoter region (-42, -32, -13ins).

ST	CTX-M	ESBL	AmpC plasmid	AmpC chrom	Other	Total
131	99	14	1	6	0	120
73	1	1	0	15	0	17
12	0	0	1	12	0	13
38	1	8	2	0	0	11
69	3	4	2	0	0	9
648	3	0	3	1	1	8
405	5	1	0	0	1	7
88	3	0	0	2	0	5
778	0	0	4	0	0	4
10	1	0	1	0	0	2
23	1	1	0	0	0	2
40	0	0	0	2	0	2
58	0	2	0	0	0	2
95	1	0	1	0	0	2
127	0	0	1	1	0	2
141	2	0	0	0	0	2
357	0	0	0	2	0	2
393	2	0	0	0	0	2
617	2	0	0	0	0	2
636	1	1	0	0	0	2

6.3.3 Association of Sequence Type with AmpC Genotype

The 19 isolates carrying an AmpC plasmid genes were allocated to 14 different sequence types. Eleven of the sequence types were seen as individuals, one of which (ST5023) was novel to this study. ST778 was designated for 4 of the isolates, ST38 for 2 isolates and ST69 for 2 isolates.

The most common *ampC* chromosomal mutation was the -32 mutation, seen in a total of 36 isolates. These isolates were allocated to 9 different sequence types. The

majority were designated as ST73 (38.9%), ST12 (33.3%), ST131 (8.3%) and ST357 (5.6%). The remaining five isolates were allocated to individual sequence types.

The association of sequence type with the allocated AmpC phylogroups from Chapter 5 is provided in **Table 6-4**. The most common sequence type (ST131) was seen in 120 isolates. Within this group, there were five different AmpC phylogroups recorded (G9, G13, G22, G26 and G32). Of these, G9 was the predominant phylogroup present, representing 113 isolates (94.2%). This suggests that 42% of all the 275 isolates tested for both MLST profiles and AmpC chromosomal genotypes had identical sequences across a combined length of over 3600 nucleotides (nearly 0.1% of the whole genome for *E. coli*). The seven isolates with one of the other four phylogroups in the ST131 group were all in the Non-ESBL group, and all of which had one of the three key *ampC* chromosomal mutations present (-42, -32 or -13ins).

6.3.4 Detection of Novel Sequence Types

Five isolates (DO-015, G-394, G-430, G-447 and TA-034) gave sequences for one locus each that did not have a recognised allele number. Full sequence type profiles were therefore not available for these five isolates. The raw sequence data files for the five alleles were submitted to the database curator for allocation of new allele numbers. Following the acceptance of the data files and the allocation of new allele numbers, these five isolates were registered as sequence types ST5025 to ST5029.

A further five isolates (DO-013, G-355, G-364, TA-040, TR-050) had valid allele numbers at the seven loci, but combinations of which had not previously been registered on the database. These were registered on the online database, with new sequence types generated as ST5015, ST5016, ST5017, ST5023 and ST5024, respectively. Overall, therefore, this study discovered ten new sequence types, all of which were registered on the central curated database (www.mlst.warwick.ac.uk/mlst/dbs/Ecoli).

Table 6-4: Distribution of *E. coli* sequence types (ST) by different AmpC phylogroup.
Only the most common 12 sequence types are shown, with the relevant AmpC phylogroups. The AmpC phylogroups were allocated in Chapter 5.

Sequence Type	G0	G1	G2	G4	G5	G8	G9	G13	G17	G19	G20	G21	G22	G26	G27	G29	G32	G33	G35	G36	G37	G38	neg	Total
131							113	2					2	1			1						1	120
73			11	1											1			1	1	2				17
12		12									1													13
38										11														11
69										8													1	9
648									4			3				1								8
405																					6	1		7
88					2	3																		5
778										4														4
393																						3		3
617	3																							3
636																				3				3

6.3.5 Comparison of Diversity Indices

The Simpson's Index of Diversity (Hunter & Gaston, 1988) is an indication of the diversity of a particular population. It represents the probability that two isolates selected at random from the same population are truly different. A population in which every isolate is different would have a diversity index of 1.0. The Diversity Index (DI) for the whole population was 0.789, suggesting that 78.9% of the isolates were different. Confidence intervals (95%) of 0.737 to 0.841 where calculated using the method of Grundmann *et al*. (2001).

In the two main groups of isolates (ESBL and Non-ESBL), there were clear differences in the ST patterns. The Non-ESBL group, comprising mainly of AmpC-producing isolates, had 73 with a total of 29 different sequence types. The most common sequence type was ST73 (20.5%), with ST12 the next common (19.2%). In this group, the ST131 isolates represented only 9.6% of the isolates (n=7). The Non-ESBL group had a DI of 0.91 (95% CI: 0.87-0.95), indicating a population with a high degree of diversity.

The larger ESBL group, comprising 196 isolates, had a total of 52 different sequence types. The most common sequence type by far was ST131, with 114 isolates (58.2%). The next common sequence type was ST405, with only seven (3.6%) isolates. The presence of the dominant ST131 clone, meant the overall DI for this group was 0.66 (95%CI: 0.58-0.74), representing a significantly less diverse population than the Non-ESBL group.

The overall populations for the five laboratories showed some variation in the diversity indices (**Figure 6-1**). Although not significantly different from the mean for the whole group, the diversity index for the Truro laboratory was significantly different to the indices for both Dorchester and Gloucester, at the 95% confidence interval.

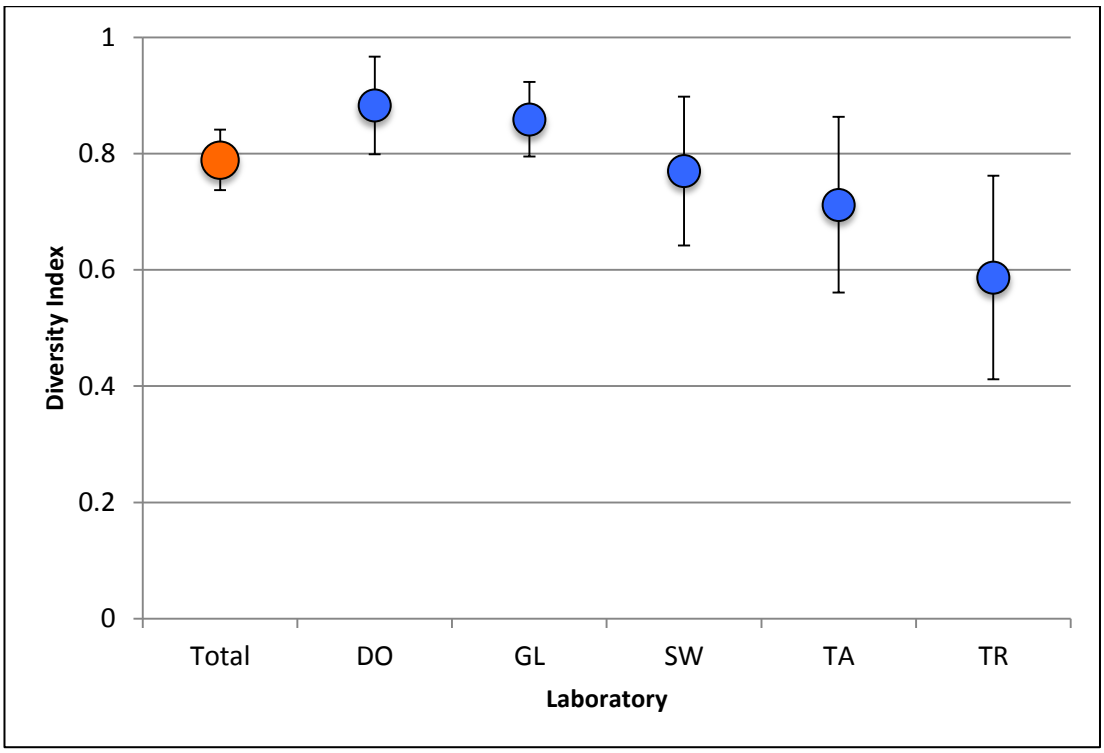

Figure 6-1: Comparison of Diversity Indices (DI), with 95% confidence intervals, for the five laboratories in the regional study.
The five laboratories are shown in blue. The overall DI for the whole population is shown in red. Lab Key: Dorchester (DO), Gloucester (GL), Swindon (SW), Taunton (TA) and Truro (TR).

6.3.6 Phylogenetic Analysis of Sequence Types

At the time of checking (27th August 2015), there were 5216 ST profiles for *E. coli* and 7749 isolates registered in the principal database. Registered isolates were from a range of sources, including human, animal, food and environmental. The ten isolates from this study with novel sequence types are included in these totals.

The 275 isolates from the current study were analysed with the eBURST software. There were 74 different sequence types allocated into 16 groups with 33 isolates appearing as singletons (**Figure 6-2**). The largest group included 18 isolates and 5 sequence types. ST38 was designated as the group founder by the eBURST software. The other four STs (ST315, ST778, ST963 and ST1177) in the group were all single-locus variants (SLV).

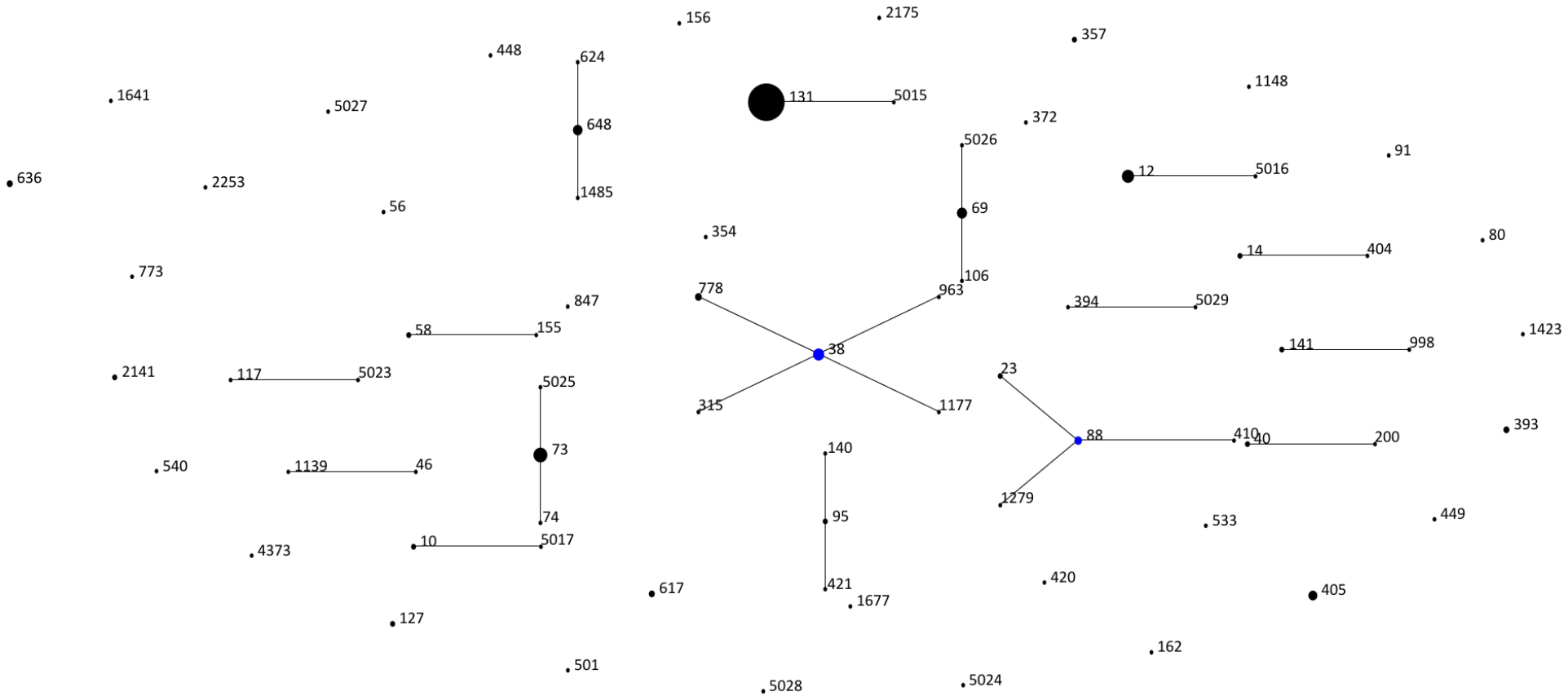

Figure 6-2: eBURST diagram for the 275 *E. coli* isolates in the regional study with a complete sequence type profile.
The size of each circle is proportionate to the number of isolates in that ST. The blue circles represent the two main founder STs, as determined by the eBURST software (Feil *et al.*, 2004).

Other groups with designated founders included Group ST88, with 9 isolates and 4 sequence types. Group ST648 comprised of 10 isolates and 3 sequence types. Group ST69 had 11 isolates and three sequence types. Group ST95 had 4 isolates and three sequence types. Group ST73 was the last designated group with ≥ 3 STs, with 19 isolates and three sequence types.

The other ten groups all comprised of two sequence types with no founder identified. The most common sequence type (ST131) was shown to be represented largely by a single clone. The sequence type did have one isolate acting as a SLV; ST5015 was novel to this study and differed only at the *recA* locus.

6.4 Discussion

6.4.1 Common MLST Sequence Types

A total of 74 different sequence types were identified in the 275 isolates tested. The most common sequence type was ST131, which accounted for 43.6% of all isolates. The next most common sequence types were ST73 (6.2%), ST12 (4.7%), ST38 (4.0%) and ST69 (3.3%). Overall, 68% of isolates were allocated to one of just 10 sequence types. These findings are similar to those of others, with ST131, ST73, ST38 and ST69 commonly reported in other studies.

In a UK study (Nottingham), Croxall *et al*. (2011) found 52 sequence types in 121 *E. coli* isolates isolated from urine samples from an elderly patient population. The most common types were ST131, ST73 and ST69. In another UK study (Yorkshire), Horner *et al*. (2013) investigated 770 *E. coli* isolates from bacteraemia cases over a 2-year period. A total of 96 different sequence types were found, with just four STs accounting for 55% of the isolates. The most common types were ST73, ST131 and ST69. Adams-Sapper *et al*. (2013) investigated a series of Gram-negative bacteraemia isolates in the US. The study found that nearly two-thirds of the 249 *E. coli* isolates were allocated to just five sequence types: ST131 (23%), ST95 (18%), ST73 (8%) and ST69 (9%).

In terms of data from sensitive isolates, a European study (Bengtsson *et al.*, 2012) of 80 *E. coli* isolates from four countries (Greece, Portugal, Sweden and the UK) investigated uropathogenic isolates without any detectable antibiotic resistance. The 80 isolates were allocated to 36 different sequence types, eleven of which were new to the study. The most common STs in the sensitive isolates were ST73, ST95 and ST420.

The eBURST diagram (**Figure 6-2**) for the regional isolates demonstrate clearly the dominance of the ST131 clone, and how it sits as a separate clone within the overall population. Other than ST131, there were a number of significant sequence types by volume, including ST73, ST12, ST38, ST69 and ST648. These are broadly similar findings to those observed in other UK studies of uropathogenic isolates. Gibreel *et al*. (2012) found that ST73 was the most common, with ST69 the third most

common after ST131, in uropathogenic UK isolates with a range of antibiotic susceptibilities. Lau *et al*. (2008) found ST131 as the most common sequence type in 68 uropathogenic UK isolates with cephalosporin resistance, followed by ST73 and ST95. In these studies of UK uropathogenic isolates, ST12 was either not present, or was seen only in small numbers. In the current study, it was seen as the third most common sequence type (4.7%).

In the current study, there were two main groups of sequence types, designated as clonal complexes as they differed at just one allele. ST38 was the founder of a complex that included ST778, ST1177, ST315 and ST963. This complex included 7 isolates with an AmpC plasmid gene and 11 isolates with ESBL resistance (mainly non-CTX-M). Secondly, ST88 was the founder of a complex that included ST23, ST1279 and ST410. This group comprised mainly of isolates with confirmed CTX-M genes, with two isolates having *ampC* chromosomal mutations (-42 mutation).

6.4.2 Association of Sequence Type with Location

The distribution of ST131 varied significantly between the five locations. The Gloucester and Truro laboratories had proportions of ST131 that differed significantly from the average rate for the whole population. There was also a level of variation seen in some of the other common sequence types, and some types that were not seen at all in one or more locations. There were also significant differences seen in the diversity indices for some of the laboratories. This is probably a reflection of the dominance of the ST131 clone in the whole population. The laboratory with the lowest proportion of ST131 isolates (Dorchester) was also the one with the highest diversity index. The reverse was true for the Truro laboratory, with the highest proportion of ST131 isolates and the lowest diversity index. Again, this confirms the significance of this particular clone, and the impact it has on the diversity of a particular population. The impact of the ST131 clone dominance may have been reduced if a larger number of isolates from each laboratory had been tested.

6.4.3 Association of Sequence Type with AmpC Plasmid Genes

The 19 isolates identified as carrying AmpC plasmid genes were allocated to 14 different sequence types, with 11 seen as individuals. This confirms the findings of others, in which isolates with AmpC plasmid genes had a high degree of diversity. In one of the few studies using MLST on AmpC-producing isolates, Naseer *et al*. (2010) investigated 402 potential AmpC-producing *E. coli* isolates in Norway, collected from 21 laboratories. A total of 38 of the *E. coli* isolates were confirmed as carrying an AmpC plasmid gene (35/38 were CMY-2), and were allocated to 25 different sequence types.

In a Spanish study, Gude *et al.* (2013) investigated 72 *E. coli* isolates known to be carrying AmpC plasmid genes (mainly CMY-2). Although the study used PFGE for typing purposes, the data showed 59 different clones amongst the isolates. The authors suggested that there is likely to be a wide heterogeneity of clones and that the AmpC plasmid-mediated mechanism is not due to the dissemination of a clonal strain.

Although in the current study there were 14 different STs identified in the 19 isolates with AmpC plasmid genes, the ST778 isolates were exclusively linked with the carriage of an AmpC plasmid gene (CIT-type). The numbers were small, however, with only four isolates, and were only isolated in the Dorchester and Gloucester laboratories. It is not clear, without testing a larger number of isolates, whether these would be seen elsewhere in the region.

6.4.4 Association of Sequence Type with *ampC* Chromosomal Mutations

Published studies using MLST to investigate isolates with *ampC* chromosomal mutations are rare. In addition to the paper by Guillouzouic *et al.* (2009), a more recent study from the Netherlands investigated the carriage of ESBL and AmpC genes in rectal swabs (van Hoek *et al.*, 2015a). The 8 isolates with *ampC* chromosomal mutations were designated as sequence types ST131 (n=2), ST453 (n=2), ST88, ST95, ST345 and ST500. These findings are substantially different to those in the current study, in terms of the sequence types found. The Guillouzuic

paper tested a small number of human and animal isolates, which were probably not fully representative of uropathogenic *E. coli* isolates. Similarly, van Hoek *et al*. tested isolates that had been isolated from faecal screening samples. It is possible that these would have been different in terms of virulence and pathogenicity, and represent a totally different population.

The comparison of MLST data with *ampC* chromosomal mutations has not previously been published for UK strains, or performed on the scale seen in the current study. The -32 mutation was seen in isolates assigned mainly to ST73 (n=14) and ST12 (n=12). These two sequence types were the most common overall, after the ST131 isolates. It is possible that these represent the presence of a dominant clone with chromosomal *ampC* mutations. However, one of these sequence types (ST73) is commonly found in sensitive strains, so may simply reflect normal strains with spontaneous mutations. Bengtsson *et al*. (2012) investigated 80 uropathogenic isolates from four countries (Greece, Portugal, Sweden and the UK). All isolates were selected on the basis of having no detectable antibiotic resistance. The three most common sequence types included ST73. There were only three isolates of ST12, all of which were isolated in Greece. The ST12 isolates from the current study may represent a potentially sub-dominant clone of *E. coli*, which is genotypically characterised by the -32 *ampC* promoter mutation.

6.4.5 Comparison of Diversity Indices

It was clear in the current study that there were significantly different diversity indices for the two main groups of isolates (ESBL and Non-ESBL), at 66% versus 91%. This is almost certainly a reflection of the dominance of the ST131 clone in the ESBL population.

In a study of rectal screening swabs, Izdebski *et al*. (2013) investigated 376 *E. coli* isolates collected in five centres in four countries (France, Italy, Spain and Israel). A total of 76 STs were identified from the 240 isolates that were investigated by MLST. The diversity index of the whole group was 81.9% (95% CI of 77.9 to 85.8), but ranged from 73.8% to 91.3% between centres. The authors commented that approximately half of the sequence types observed in a centre were usually not

observed in the others. A similar level of variance in the diversity indices for the five laboratories in the current study was also observed, ranging from 58.7% (Truro) to 88.3% (Dorchester). This variation in diversity is partly due to the dominance of the ST131 clone, but may also be due to natural variations in the local populations.

Adams-Sapper *et al*. (2013) found that the overall diversity index for a population of 249 bacteraemia *E. coli* isolates was 89%. Although there was some difference in the diversity indices for sensitive and resistant isolates (87% and 81%, respectively), this was not found to be statistically significant. Lau *et al*. (2008) established that there were differences in the diversities of different types of populations. For example, *E. coli* isolates from urine samples were more diverse than blood culture samples, and isolates from the community were more diverse than those isolated from hospital patients. The authors suggest that ST131 could be more likely to be exposed to antibiotics, resulting in the development of virulence and antibiotic resistance over time. Woodford *et al*. (2011) concur, suggesting that those clones that are circulating widely are more likely to acquire the resistance-associated plasmids that are present locally.

In the current study, CTX-M genes were found in 56 isolates assigned to 39 different sequence types. Eight of these 39 sequence types were novel to this study. This confirms that CTX-M genes are not exclusively limited to the ST131 isolates, and suggests that a range of uropathogenic strains can acquire the CTX-M genes via plasmids.

6.5 Summary

The current study provides data that confirms the dominance of the ST131 clone as a major cause of cephalosporin resistance in the South West region. This is clear from the fact that 43.6% of all isolates were of this particular sequence type, and that 82.5% of the ST131 isolates were confirmed as having $bla_{\text{CTX-M}}$ genes. There was also evidence that the proportions of this dominant clone vary between the five laboratories. Although this may have been due to the study design, and the restricted number of isolates collected from each laboratory, it may also be due to

differences in other local factors (such as antibiotic prescribing polices producing a selective pressure on strains).

The ST131 isolates were predominantly found to have ESBL genes present, although this was not exclusive. The next most common sequence types found (ST73 and ST12) had a strong association with the presence of *ampC* chromosomal mutations. This suggests that there may be a sub-dominant population of *E. coli* strains with *ampC* mutations in the South West. Previous studies have reported a low prevalence of ST12 in uropathogenic isolates. The presence of a sub-dominant ST12 clone with the -32 *ampC* mutation would also explain the higher prevalence of this mutation in the study compared to the findings of others. Overall, however, the diversity of the group of isolates with AmpC mechanisms (chromosomal mutations or plasmid genes) was significantly greater than that ESBL group, suggesting that a dominant clone is less likely.

It was interesting to note that the 19 isolates with AmpC plasmid genes present were assigned to 14 different sequence types. This suggests that the plasmid-carrying isolates are not the result of the spread of a dominant clone, rather the genuine acquisition of plasmids. The potential importance of the four ST778 isolates with a CIT-type plasmid gene present will need to be investigated further. It also seemed that the isolates with AmpC plasmid genes were more closely related to isolates with bla_{ESBL} genes, than those with *ampC* mutations.

The current study identified ten new sequence types from the 275 isolates tested. The five new allele sequences identified were all single base pair mutations. This confirms the utility of the method as a broad typing method, as small changes in the housekeeping genes can be detected and used to differentiate strains and changes in populations over time. The current study has made a modest, but important, contribution to the development and utility of MLST as an international typing method.

6.6 Future Work

The majority of the isolates included in the current study originated from urine samples. Whilst this has provided a robust group to investigate, it has excluded a significant source of pathogenic *E. coli* isolates. Some of the *E. coli* MLST data published have identified differences between the diversity indices between populations of uropathogenic isolates and bacteraemia isolates, even though the underlying lineages should be similar (Lau *et al.*, 2008). It would be useful to expand the current study to include a larger group of bacteraemia isolates. National data indicate that there are approximately 260 strains of *E. coli* isolated from blood cultures in the South West every month (Public Health England, 2015b). This would also help to confirm the significance of the four ST778 isolates observed exclusively with an AmpC plasmid gene.

The second and third most common sequence types were ST73 and ST12, both of which were associated with the -32 chromosomal *ampC* mutation. Although there is some evidence to suggest that sequence type ST73 is common in uropathogenic isolates, this has not been confirmed in the local populations. Investigating a selection of cephalosporin-susceptible isolates from each of the five laboratories would confirm both the presence of chromosomal *ampC* mutations in sensitive isolates and the importance of the ST73 and ST12 clones.

The use of MLST in this study has provided a good overall picture of the cephalosporin-resistant *E. coli* population in five locations in the South West of England. The main benefit of using this method is that results are directly comparable with the work of others. However, the resolution is not sufficient to confirm the presence of cross-infection and direct transfer of strains between patients. There were 12 isolates of sequence type ST12, all of which had the same *ampC* chromosomal mutation pattern. Further investigation with a more discriminatory typing method, such as single nucleotide polymorphism (SNP) analysis (Sherry *et al.*, 2013) or ribosomal MLST (Jolley *et al.*, 2012), would offer a greater insight of how strains transfer between patients.

7 Conclusion

7.1 Genotypic Characteristics of AmpC-Producing *E. coli*

This study presents a unique picture of AmpC-mediated resistance in *E. coli* isolates in the South West of England. It also represents a significant contribution to the overall understanding of chromosomal *ampC* mutations in UK isolates. There are no other published studies of *ampC* chromosomal mutations in UK *E. coli* isolates on this scale, or that investigate differences between populations. In the 276 isolates collected in the regional study, 25.8% were confirmed as having a genotype associated with AmpC resistance. This is significantly higher than the proportion (7.1%) found by Potz *et al*. (2006) ($p<0.05$, Fisher's Exact Test). However, the AmpC mechanisms in their isolates were not fully investigated, with 26% being allocated to the "other" category. It is likely that many of these would have had *ampC* chromosomal mutations.

There were 19 isolates in the regional study found to be carrying an AmpC plasmid gene. The AmpC plasmid genes in the CIT group were likely to be CMY-2, but this was not fully confirmed. If true, this would confirm the importance of this particular mechanism for AmpC enzyme production. In this study, only the CIT and DHA types of AmpC plasmid genes were found. It is possible that small numbers of other plasmid gene types would be found in the South West if further isolates or laboratory locations were tested. However, the potential additional impact of these strains would be minimal.

The analysis of *ampC* chromosomal mutations is complicated by the numbers of different mutations present. In the regional study, there were mutations in 28 different positions in the amplified region, giving 31 different phylogroups (**Table 5-6**). A similarly complex picture was seen by Mulvey *et al.* (2005). Not all of the phylogroups were associated with AmpC resistance. Half of the phylogroups contained one of the key mutations (-42, -32, -13ins) described by Tracz *et al.* (2007). The other half were associated mainly with ESBL resistance. Although not investigated in this study, other studies have found some *ampC* chromosomal mutations in *E. coli* isolates without detectable resistance (Corvec *et al.*, 2007).

Overall the three key *ampC* mutations (-42, -32 and -13ins) were found in 18.6% of the cefpodoxime-resistant isolates collected in the five laboratories. So although a smaller proportion compared to the dominance of the ESBL isolates, chromosomal *ampC* resistance is still accounting for nearly a fifth of cephalosporin resistance in *E. coli.*

There were two key findings from the regional stage of the project. Firstly, that the -32 (T to A) mutation was more common than the -42 and -13ins mutations. Although this was also seen in the pilot study, it was in direct contrast to all other studies of *ampC* chromosomal mutations in *E. coli*. The second interesting finding was the presence of a mutation (thymine base insertion) at position -20 (**Figure 5-9**). This was only found in two isolates, but has not been previously reported in the UK. As this mutation would increase the inter-box distance by 1bp to the optimum distance of 17bp, it is likely to have a similar impact as the -13ins mutation on AmpC enzyme production. It should, therefore, be considered as a fourth key chromosomal *ampC* mutation.

7.2 Existence of a Dominant AmpC-Producing *E. coli* Clone

The impact of ST131 as a dominant clone was clearly evident in this study. Of all the isolates tested, 43.6% were of this sequence type (**Table 6-1**). The evidence for a second dominant clone was less convincing. In the 19 isolates with AmpC plasmid genes, 11 were of different sequence types; suggesting a reasonable amount of diversity within this group. There were four isolates of sequence type ST778 that were all found to be carrying an AmpC plasmid gene (CIT type). It is not clear whether these were all the same clone, or simply strains of the same sequence type that had acquired the AmpC plasmid genes independently. Cross-infection of the same strain between patients may have been a consideration if all had been collected in the same laboratory. Similarly, a failure of the de-duplication stage of the collection process to detect the same strain from the same patient in multiple samples could also explain a cluster of identical isolates in one laboratory. Even if these four isolates do represent a single clone spreading across the region, the current impact on the overall population is small.

In terms of strains with chromosomal *ampC* mutations, there were 12 isolates of sequence type ST12 that all had the same mutation at position -32 (**Table 6-4**). These isolates were isolated from three different laboratories, suggesting again that a cross-infection or outbreak event was not the cause. If these were all a single clone that had spread across part of the region, it would still represent a fairly small impact compared to that of the ST131 clone (4.4% versus 43.6%). The presence of a dominant clone, however, would explain why the -32 mutation was more prevalent in this study compared to others. This was the finding in both the pilot study and the regional study, which were conducted two years apart.

7.3 Utility of SYBR Green Real-Time PCR

This study used SYBR Green chemistry as the method for detecting a PCR product. Although the real-time PCR process could have been developed further to include the use of fluorescent probes, this would have added a further layer of method optimisation and cost. The results of this study demonstrate that a robust method for detecting the presence of PCR products can be implemented on equipment commonly found in clinical laboratories.

Since the conclusion of this study, the same underlying method and equipment have been used in the Gloucester laboratory to detect a number of other genes, using primers published by others. These have included fosfomycin (*fosA*) resistance genes in Enterobacteriaceae (Gray *et al.*, 2001), MLST genes for typing *Clostridium difficile* (Griffiths *et al.*, 2010), *spa* typing genes in *Staphylococcus aureus* (Harmsen *et al.*, 2003) and the 16S ribosomal genes used in bacterial identification (Harris & Hartley, 2003). All of these have been successfully implemented in the laboratory, using the same protocols and regents as in the current study. This confirms the potential usefulness of the method as a research and diagnostic tool for clinical laboratories.

7.4 Differences in Laboratory Populations

In terms of differences between laboratories, there were significant variations in the numbers of isolates with AmpC plasmid genes collected from laboratories

(**Figure 5-7**). This ranged from 0% in Truro to nearly 20% in Dorchester. There were variations also in the proportion of isolates with a -32 chromosomal mutations, ranging from 2.2% in Dorchester to 19% in Gloucester. The underlying reasons for these variations were not investigated further. One reason could be due to differences in antibiotic prescribing policies. Although no direct evidence exists for this in relation to urinary tract infections, there is some general evidence that prescribing rates do differ amongst primary care providers (Public Health England, 2014).

The differences seen in prevalence rates of AmpC plasmid-carrying isolates may, however, just be a factor of study design. Laboratories were asked to collect up to 50 isolates over a period of 3 months. It may be that the data are unusual for these laboratories, and would not be replicated if re-investigated. The laboratory (Dorchester) with a high AmpC plasmid gene prevalence rate had a low rate for CTX-M-carrying ST131 isolates. This was also the reverse picture for the Truro laboratory. It is possible that the presence of the dominant ST131 clone is exerting a selective pressure, limiting the isolation of other resistance mechanisms.

7.5 Study Limitations

There are a number of limitations relating to the range of laboratories participating in the study. Although the five laboratories represented a good geographic coverage of the South West region, some of the larger laboratories were not able to participate. A particularly notable absence is Bristol, which would have provided a selection of isolates from a more urban area, when compared to laboratories such as Truro and Taunton. This limitation is due to the availability of resources in clinical laboratories to participate in research studies. Although laboratories were offered financial support of £3 per isolate submitted, this would only have covered basic time and materials costs.

To meet timeframes and to help secure laboratory support, a relatively short collection period was used, and laboratories were asked to collect only the first 50 isolates. This may have limited the number of different types of resistance mechanisms seen, and would have increased the impact of any outbreak or cross-

infection event occurring at the time of collection. The significant differences between the Truro and Dorchester isolates may just have been a temporary shift in strain incidence, or may actually represent a difference in selective pressure from local prescribing policies. This can only be confirmed with the collection of further isolates, preferably over multiple time points.

The limited collection period also meant that few isolates from bacteraemia cases were collected. These are the strains that have the highest impact on morbidity and mortality in patients. However, the study did collect 97% of the isolates in the regional stage from urine samples. This provides a robust population of isolates from similar types of infection, and also enables isolates to be compared with other studies and with the results of the pilot study. It is known that 45% of *E. coli* sepsis cases have the urinary tract as a suspected origin source (Davies *et al.*, 2012), so investigating a population of >250 uropathogenic isolates still remains valid. These are also the strains most likely to circulating within populations as pathogenic strains.

Focusing on pathogenic isolates, however, brings its own problems. Spratt and Maiden (1999) suggest that there is an inherent bias of any study of this nature, in which the focus is on disease-causing strains. In these cases, selecting the more virulent pathogenic strains may be neglecting the less pathogenic commensal or environmental strains, which often comprise a higher proportion of the overall population. The authors also suggest that restricting analysis to pathogenic isolates will underestimate the diversity of a population and overestimate the clonality. Designing a study to investigate commensal *E. coli* strains is possible, but often relies on the success of screening faeces sample or rectal swabs from healthy volunteers. This type of study has inherent difficulties with ethics approval and securing decent participating rates. It is difficult to use other types of samples routinely submitted to clinical laboratories, as even clinical faeces samples would be submitted from patients for the investigation of gastro-intestinal problems.

7.6 Contributions to New Knowledge

The contributions to new knowledge are four-fold. Firstly, the study confirmed that SYBR Green Real-Time PCR can be used, and easily adapted, for detecting antibiotic resistance genes and typing genes in *E. coli.* Although this would be limited to research use only, it nonetheless offers most clinical laboratories an easy way of introducing PCR and sequencing methods into research projects. The use of melting curve analysis means that the PCR product can be detected and partly characterised without the need for a separate gel electrophoresis stage.

Secondly, the study made a direct contribution to the continuing development of the *E. coli* MLST scheme, by registering five new alleles and ten new sequence types. The detection of novel sequence types in the current study confirms the usefulness of this method in analysing bacterial populations, as small changes to alleles would be expected to occur naturally over time.

Thirdly, the study confirmed the findings of others that *E. coli* strains with AmpC plasmid genes are a diverse group. There was no significant evidence that a dominant clone of AmpC plasmid-carrying *E. coli* exists within the South West region. This suggests that isolates with AmpC plasmid genes are pathogenic strains that have acquired the plasmids on an ad-hoc or opportunistic basis, and provides some reassurance against the concerns of these clones spreading like ST131.

Finally, the study identified some potential evidence of spread of an *E. coli* clone with a mutation in the *ampC* promoter region. In contrast to other studies, the current study identified a higher number of isolates with the -32 mutation present in both the pilot study phase and regional study phase. On investigation with MLST, there were 12 isolates of ST12 which all had the same AmpC genotype. Whether this is truly a dominant clone of *E. coli* can only be determined with further investigation of additional isolates.

7.7 Recommendations for Future Work

This study set out to achieve four key objectives. In doing so, it has generated a comprehensive picture of cephalosporin-resistance in the South West of England.

This picture is by no means complete, however. There are a number of natural avenues of further work and new questions that need answering.

One obvious recommendation would be to repeat the study at a different time point. This would confirm the key findings of this study, and help collect more of the unusual strains (e.g. ST778 with AmpC plasmid genes and ST12 with -32 mutations). Restricting a future study to only collecting bacteraemia isolates would provide an important dataset of isolates that have progressed to causing sepsis. This could be developed further to include clinical and risk factor data collection.

The evidence for clonal spread of AmpC resistance should be investigated further. The four isolates of ST778 with AmpC plasmid genes and the twelve isolates of ST12 with the same *ampC* chromosomal mutation could be investigated using whole genome data. Ribosomal MLST (Jolley *et al.*, 2012) would, in the first instance, be able to differentiate isolates were possible. To confirm whether isolates were truly identical clones, however, one would need to use a more details analysis of the genome such as SNP analysis (Sherry *et al.*, 2013).

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9 Appendices

9.1 Quantification of DNA from extraction process

Table 9-1: Quantification of DNA from extraction process
DNA quantification in crude lysates was done using a Qubit fluorometer, testing a sample volume of 10µl. Figures are given in ng/µl**.**

	Test 1	Test 2	Test 3	Average
Sample 1	1.73	1.67	1.66	1.69
Sample 2	8.44	8.38	8.32	8.38
Sample 3	2.72	2.70	2.68	2.70
Sample 4	0.76	0.74	0.73	0.74
Sample 5	1.18	1.18	1.17	1.18
Sample 6	2.60	2.60	2.58	2.59
Sample 7	0.50	0.49	0.48	0.49
Sample 8	3.06	3.06	3.04	3.05
Sample 9	1.00	0.97	0.97	0.98
Sample 10	2.40	2.38	2.36	2.38

9.2 Full list of AmpC plasmid genes

Full list of AmpC plasmid genes available on the website (www.lahey.org/studies/) at the time of last checking (2nd February 2015).

Enzyme	Nucleotide		Enzyme	Nucleotide
CMY-1	X92508		CMY-117	KM087844
CMY-2	X91840		CMY-118	KM087838
CMY-3			CMY-119	KM087845
CMY-4	Y15130		CMY-120	Assigned
CMY-5	Y17716		CMY-121	Assigned
CMY-6	AJ011293		CMY-122	Assigned
CMY-7	AJ011291		CMY-123	Assigned
CMY-8	AF167990		CMY-124	Assigned
CMY-9	AB061794		CMY-125	Assigned
CMY-10	AF357597		CMY-126	Assigned
CMY-11	AF357599		CMY-127	Assigned
CMY-12	Y16785		CMY-128	Assigned
CMY-13	AY339625		CMY-129	Assigned
CMY-14	AJ555825		CMY-130	Assigned
CMY-15	AJ555823		CMY-131	KP281294
CMY-16	AJ781421		CMY-132	KP862820
CMY-17	AY513266		CMY-133	KP862819
CMY-18	AY743434		CMY-134	Assigned
CMY-19	AB194410		CMY-135	KP981366
CMY-20	AY960293		CMY-136	Assigned
CMY-21	DQ139328		ACC-1	AJ133121
CMY-22	DQ256079		ACC-2	AF180952
CMY-23	DQ463751		ACC-3	AF180958
CMY-24	EF415650		ACC-4	EF504260
CMY-25	EU515249		ACC-5	HE819401
CMY-26	AB300358		ACT-1	U58495
CMY-27	EU515250		ACT-2	AM076977
CMY-28	EF561644		ACT-3	EF125013
CMY-29	EF685371		ACT-4	EU427302
CMY-30	EF685372		ACT-5	FJ237369
CMY-31	EF622224		ACT-6	FJ237366
CMY-32	EU496815		ACT-7	FJ237368
CMY-33	EU496816		ACT-8	Assigned
CMY-34	EF394370		ACT-9	HQ693810
CMY-35	EF394371		ACT-10	JN848330
CMY-36	EU331426		ACT-11	Assigned

Enzyme	Nucleotide		Enzyme	Nucleotide
CMY-37	AB280919		ACT-12	JX440355
CMY-38	AM931008		ACT-13	HE819402
CMY-39	AB372224		ACT-14	JX440354
CMY-40	EU515251		ACT-15	JX440356
CMY-41	AB429270		ACT-16	AB737978
CMY-42	HM146927		ACT-17	KF992026
CMY-43	FJ360626		ACT-18	KF992028
CMY-44	FJ437066		ACT-19	KF992029
CMY-45	FN546177		ACT-20	KF526117
CMY-46	FN556186		ACT-21	KF526118
CMY-47	HM046998		ACT-22	KF992027
CMY-48	HM569226		ACT-23	KF515536
CMY-49	GQ402541		ACT-24	KJ207207
CMY-50	FN645444		ACT-25	KJ207208
CMY-51	JQ733571		ACT-26	Withdrawn
CMY-52	Assigned		ACT-27	KJ207209
CMY-53	HQ336940		ACT-28	KJ207206
CMY-54	HM544039		ACT-29	KM087832
CMY-55	HM544040		ACT-30	KM087833
CMY-56	HQ322613		ACT-31	KM087843
CMY-57	HQ285243		ACT-32	KM087835
CMY-58	HQ185697		ACT-33	KM987834
CMY-59	AB587082		ACT-34	Assigned
CMY-60	JF460794		ACT-35	LC004922
CMY-61	JF460795		ACT-36	KM926621
CMY-62	JF460796		ACT-37	KM926622
CMY-63	HQ650104		ACT-38	KP836350
CMY-64	HQ832678		CFE-1	AB107899
CMY-65	JF780936		DHA-1	Y16410
CMY-66	JN714478		DHA-2	AF259520
CMY-67	JQ711185		DHA-3	AY494945
CMY-68	JN714480		DHA-4	Assigned
CMY-69	JX049132		DHA-5	JF273491
CMY-70	JX440350		DHA-6	HQ322612
CMY-71	JQ711184		DHA-7	HQ456945
CMY-72	JX440352		DHA-8	Assigned
CMY-73	GQ351345		DHA-9	KJ207201
CMY-74	JX440349		DHA-10	KP050490
CMY-75	JQ733572		DHA-11	Assigned
CMY-76	JQ733573		DHA-12	HG798963
CMY-77	JX440353		DHA-13	KM087855

Enzyme	Nucleotide		Enzyme	Nucleotide
CMY-78	JQ733575		DHA-14	KM087854
CMY-79	JQ733576		DHA-15	KM087853
CMY-80	JQ733577		DHA-16	KM087852
CMY-81	JQ733578		DHA-17	KM087850
CMY-82	KJ207203		DHA-18	KM087841
CMY-83	JX440351		DHA-19	KM087849
CMY-84	JQ733579		DHA-20	KM087848
CMY-85	KJ207202		DHA-21	KM087847
CMY-86	KJ207204		DHA-22	KM087856
CMY-87	AB699171		DHA-23	Assigned
CMY-88	Assigned		FOX-1	X77455
CMY-89	Assigned		FOX-2	Y10282
CMY-90	HE819404		FOX-3	Y11068
CMY-91	Assigned		FOX-4	AJ277535
CMY-92	Assigned		FOX-5	AY007369
CMY-93	KF992025		FOX-6	AY034848
CMY-94	JX514368		FOX-7	AJ703795
CMY-95	JX514369		FOX-8	HM565917
CMY-96	Assigned		FOX-9	JF896803
CMY-97	Assigned		FOX-10	JX049131
CMY-98	KC603538		FOX-11	Assigned
CMY-99	KF305673		FOX-12	Assigned
CMY-100	KF526113		LAT-1	X78117
CMY-101	KF526114		MIR-1	M37839
CMY-102	KF526115		MIR-2	AY227752
CMY-103	KF526116		MIR-3	AY743435
CMY-104	KF150216		MIR-4	EF417572
CMY-105	KJ207205		MIR-5	FJ237367
CMY-106	KM983294		MIR-6	JQ664733
CMY-107	Assigned		MIR-7	KJ207200
CMY-108	KF564648		MIR-8	KP050484
CMY-109	Assigned		MIR-9	KM087860
CMY-110	AB872957		MIR-10	KM087858
CMY-111	KJ155695		MIR-11	KM087859
CMY-112	KM087837		MIR-12	KM087863
CMY-113	KM087836		MIR-13	KM087862
CMY-114	KM087846		MIR-14	KM087864
CMY-115	KM087839		MIR-15	KM087851
CMY-116	KM087840		MIR-16	KM087861
MIR-17	LN515535		MOX-4	FJ262599
MIR-18	Assigned		MOX-5	GQ152600

Enzyme	Nucleotide		Enzyme	Nucleotide
MOX-1	D13304		MOX-6	GQ152601
MOX-2	AJ276453		MOX-7	GQ152602
MOX-3	EU515248		MOX-8	JX173956

9.3 Laboratory information sheet and submission instructions

AmpC Study

Background

The main objective of the study is to establish the regional incidence of AmpC beta-lactamase production in clinical *E. coli* isolates. The study will use real-time PCR to identify the types of AmpC enzymes present and the phylogenetic types of positive isolates. The study will also use MLST (multi locus sequence typing) to determine the types of strains circulating in the region.

The first collection period is due to run from 2nd April 2013.

Anonymised Isolates

In order to meet the requirements of the ethics panel, all isolates need to be anonymised before inclusion in the study. This ensures that each isolate is no longer linked to a patient. The study will use unique study numbers to identify isolates instead of lab numbers.

De-duplication

Before inclusion in the study, isolates should be checked to see whether a duplicate has been reported on the same patient in the previous 30 days. Duplicate isolates should not be included in the study.

Inclusion Process

Isolates are eligible for inclusion in the study if they:

- Have been identified as *E. coli* (chromogenic plate or API)
- Are cefpodoxime resistant
- Have not been previously isolated in the last 30 days

Isolates eligible for inclusion should be sub-cultured onto a nutrient agar slope. The slope should be labelled with a unique study number. No other information (e.g. surname or lab number) should be written on the slope. The corresponding sample data should be entered onto the record sheet.

Slopes should be incubated overnight, and then stored until returning to the Gloucester laboratory.

9.4 Laboratory isolate record sheet

AmpC Study

Eligibility Criteria

The study is open to all isolates of ***E. coli*** that are **cefpodoxime-resistant.**

De-duplication

Please check whether the isolate has been previously reported on the same patient in the previous 30 days.

Subculture

Please subculture the organism to a NA slope labelled **only** with a study label. Enter the relevant details onto the table below.

Study No	Specimen Type	Specimen Date	DOB	Gender	Inpatient / GP

9.5 Sequences of AmpC plasmid gene products

9.5.1 DHA Plasmid Gene

```
CT-03-DHA_DHA-F             ------------AAAATACCAGCCGGAGCTGGCTCTGCCGCAGTGGAAGG 38
CT-04-DHA_DHA-F             TGATCCGGCGGCAAAATACCAGCCGGAGCTGGCTCTGCCGCAGTGGAAGG 55
gi|2853120|emb|Y16410.1|    TGATCCGGCGGCAAAATACCAGCCGGAGCTGGCTCTGCCGCAGTGGAAGG 1350
                                         **************************************

CT-03-DHA_DHA-F             GGATCACATTGCTGGATCTGGCTACCTATACCGCAGGCGGACTGCCGTTA 88
CT-04-DHA_DHA-F             GGATCACATTGCTGGATCTGGCTACCTATACCGCAGGCGGACTGCCGTTA 105
gi|2853120|emb|Y16410.1|    GGATCACATTGCTGGATCTGGCTACCTATACCGCAGGCGGACTGCCGTTA 1400
                            **************************************************

CT-03-DHA_DHA-F             CAGGTGCCGGATGCGGTAAAAAGCCGTGCGGATCTGCTGAATTTCTATCA 138
CT-04-DHA_DHA-F             CAGGTGCCGGATGCGGTAAAAAGCCGTGCGGATCTGCTGAATTTCTATCA 155
gi|2853120|emb|Y16410.1|    CAGGTGCCGGATGCGGTAAAAAGCCGTGCGGATCTGCTGAATTTCTATCA 1450
                            **************************************************

CT-03-DHA_DHA-F             GCAGTGGCAGCCGTCCCGGAAACCGGGCGATATGCGTCTGTATGCAAACA 188
CT-04-DHA_DHA-F             GCAGTGGCAGCCGTCCCGGAAACCGGGCGATATGCGTCTGTATGCAAACA 205
gi|2853120|emb|Y16410.1|    GCAGTGGCAGCCGTCCCGGAAACCGGGCGATATGCGTCTGTATGCAAACA 1500
                            **************************************************

CT-03-DHA_DHA-F             GCAGTATCGGCCTGTTTGGTGCTCTGACCGCAAACGCGGCGGGGATGCCG 238
CT-04-DHA_DHA-F             GCAGTATCGGCCTGTTTGGTGCTCTGACCGCAAACGCGGCGGGGATGCCG 255
gi|2853120|emb|Y16410.1|    GCAGTATCGGCCTGTTTGGTGCTCTGACCGCAAACGCGGCGGGGATGCCG 1550
                            **************************************************

CT-03-DHA_DHA-F             TATGAGCAGTTGCTGACTGCACGGATCCTGGCACCGCTGGGGTTATCTCA 288
CT-04-DHA_DHA-F             TATGAGCAGTTGCTGACTGCACGGATCCTGGCACCGCTGGGGTTATCTCA 305
gi|2853120|emb|Y16410.1|    TATGAGCAGTTGCTGACTGCACGGATCCTGGCACCGCTGGGGTTATCTCA 1600
                            **************************************************

CT-03-DHA_DHA-F             CACCTTTATTACTGTGCCGGAAAGTGCGCAAAGCCAGTATGCGTACGG-- 336
CT-04-DHA_DHA-F             CACCTTTATTACTGTGCCGGAAAGTGCGCAAAGCCAGTATGCGTACGG-- 353
gi|2853120|emb|Y16410.1|    CACCTTTATTACTGTGCCGGAAAGTGCGCAAAGCCAGTATGCGTACGGTT 1650
                            ***********************************************
```

9.5.2 ACC Plasmid Gene

```
CT-01-ACC_ACC-F                AGTGGGTTCGCTGAGTAAA-CGTTTGCTGCCACCTTGGCGTCCTATGCGC 51
CT-02-ACC_ACC-F                -GTGGGTTCGCTGAGTAAA-CGTTTGCTGCCACCTTGGCGTCCTATGCGC 48
gi|5419924|emb|AJ133121.1|     AGTGGGTTCGCTGAGTAAAACGTTTGCTGCCACCTTGGCGTCCTATGCGC 950
                               ****************** ******************************

CT-01-ACC_ACC-F                AGGTGAGCGGTAAGCTGTCCTTGGATCAAAGCGTTAGCCATTACGTTCCA 101
CT-02-ACC_ACC-F                AGGTGAGCGGTAAGCTGTCCTTGGATCAAAGCGTTAGCCATTACGTTCCA 98
gi|5419924|emb|AJ133121.1|     AGGTGAGCGGTAAGCTGTCTTTGGATCAAAGCGTTAGCCATTACGTTCCA 1000
                               ****************** ******************************

CT-01-ACC_ACC-F                GAGTTGCGTGGCAGCAGCTTTGACCACGTTAGCGTACTCAATGTGGGCAC 151
CT-02-ACC_ACC-F                GAGTTGCGTGGCAGCAGCTTTGACCACGTTAGCGTACTCAATGTGGGCAC 148
gi|5419924|emb|AJ133121.1|     GAGTTGCGTGGCAGCAGCTTTGACCACGTTAGCGTACTCAATGTGGGCAC 1050
                               **************************************************

CT-01-ACC_ACC-F                GCATACCTCAGGCCTACAGCTATTTATGCCGGAAGATATTAAAAATACCA 201
CT-02-ACC_ACC-F                GCATACCTCAGGCCTACAGCTATTTATGCCGGAAGATATTAAAAATACCA 198
gi|5419924|emb|AJ133121.1|     GCATACCTCAGGCCTACAGCTATTTATGCCGGAAGATATTAAAAATACCA 1100
                               **************************************************

CT-01-ACC_ACC-F                CACAGCTGATGGCTTATCTAAAAACATGGAAACCTGCCGATGCGGCTGGA 251
CT-02-ACC_ACC-F                CACAGCTGATGGCTTATCTAAAAACATGGAAACCTGCCGATGCGGCTGGA 248
gi|5419924|emb|AJ133121.1|     CACAGCTGATGGCTTATCTAAAAGCATGGAAACCTGCCGATGCGGCTGGA 1150
                               ********************** *************************

CT-01-ACC_ACC-F                ACCCATCGCGTTTATTCCAATATCGGTACTGGTTTGCTAGGGATGATTG- 300
CT-02-ACC_ACC-F                ACCCATCGCGTTTATTCCAATATCGGTACTGGTTTGCTAGGGATGATTGG 298
gi|5419924|emb|AJ133121.1|     ACCCATCGCGTTTATTCCAATATCGGTACTGGTTTGCTAGGGATGATTG- 1199
                               **************************************************

CT-01-ACC_ACC-F                CGGCGAAAACTC-------------------------------AACTC-- 317
CT-02-ACC_ACC-F                CGGCGAAA----------------------------------------- 306
gi|5419924|emb|AJ133121.1|     CGGCGAAAAGTCTGGGTGTGAGCTATGAAGATGCGATTGAGAAAACCCTC 1249
                               ********
```

9.5.3 FOX Plasmid Gene

```
FOX-1_FOX-F                 --------CGCG-ATCGCGCTGACCCACACCGGTTTCTACTCGGTGGGAG 41
FOX-2_FOX-F                 --------------------TGACCCACACCGGTTTCTACTCGGTGGGAG 30
gi|453214|emb|X77455.1|     GCTAAAAACGCGGATCGCGCTGACCCATACCGGTTTCTACTCGGTGGGAG 1550
                                                ******* ********************

FOX-1_FOX-F                 ACATGACCCAGGGACTGGGCTGGGAGAGTTACGCCTATCCGGTGACCGAG 91
FOX-2_FOX-F                 ACATGACCCAGGGACTGGGCTGGGAGAGTTACGCCTATCCGGTGACCGAG 80
gi|453214|emb|X77455.1|     ACATGACTCAGGGGCTGGGTTGGGAGAGCTACGCCTATCCGTTGACCGAG 1600
                            ******* ***** ***** ******** ************ ********

FOX-1_FOX-F                 CAGACATTGCTGGCGGGTAACGCACCGGCGGTGAGCTTCCAGGCCAATCC 141
FOX-2_FOX-F                 CAGACATTGCTGGCGGGTAACGCACCGGCGGTGAGCTTCCAGGCCAATCC 130
gi|453214|emb|X77455.1|     CAGGCGCTGCTGGCGGGCAACTCCCCGGCGGTGAGCTTCCAGGCCAATCC 1650
                            *** *  ********** *** * **************************

FOX-1_FOX-F                 GGTTACGCGCTTTG-ATTGTGC---------------------------- 162
FOX-2_FOX-F                 GGTTACGCGCTTTA-AG---------------------------------- 146
gi|453214|emb|X77455.1|     GGTTACGCGCTTTGCGGTGCCCAAAGCGATGGGCGAGCAGCGGCTCTATA 1700
                            *************
```

9.5.4 MOX Plasmid Gene

```
MOX-1_MOX-F                 CTCA-GGATGGCAAGGCCCACTATTTCAATTACGGGGTGGCCAACCGGGA 51
MOX-2_MOX-F                 CTCA-GGATGGCA-GGCCCACTATTTCAATTACGGGGTGGCCAACCGGGA 50
gi|11761377|dbj|D13304.2|   CTCAAGGATGGCAAGGCCCACTATTTCAATTACGGGGTGGCCAACCGGGA 450
                            **** ******** ************************************

MOX-1_MOX-F                 GAGCGGGGCCAGCGTCAGCGAGCAGACCCTGTTCGAGATAGGATCCGTGA 101
MOX-2_MOX-F                 GAGCGGGGCCAGCGTCAGCGAGCAGACCCTGTTCGAGATAGGATCCGTGA 100
gi|11761377|dbj|D13304.2|   GAGCGGGGCCAGCGTCAGCGAGCAGACCCTGTTCGAGATAGGATCCGTGA 500
                            **************************************************

MOX-1_MOX-F                 GCAAGACCCTGACTGCGACCCTGGGGGCCTATGCGGTGGTCAAGGGAGCG 151
MOX-2_MOX-F                 GCAAGACCCTGACTGCGACCCTGGGGGCCTATGCGGTGGTCAAGGGAGCG 150
gi|11761377|dbj|D13304.2|   GCAAGACCCTGACTGCGACCCTGGGGGCCTATGCGGTGGTCAAGGGAGCG 550
                            **************************************************

MOX-1_MOX-F                 ATGCAGCTGGATGACAAGGCGAGCCGGCACGCGCCCTGGCTCAAGGGATC 201
MOX-2_MOX-F                 ATGCAGCTGGATGACAAGGCGAGCCGGCACGCGCCCTGGCTCAAGGGATC 200
gi|11761377|dbj|D13304.2|   ATGCAGCTGGATGACAAGGCGAGCCGGCACGCGCCCTGGCTCAAGGGATC 600
                            **************************************************

MOX-1_MOX-F                 CGTCTTTGACAGCATCACCATGGGGGAGCTTGCCACCTACAGCGCCGGAG 251
MOX-2_MOX-F                 CGTCTTTGACAGCATCACCATGGGGGAGCTTGCCACCTACAGCGCCGGAG 250
gi|11761377|dbj|D13304.2|   CGTCTTTGACAGCATCACCATGGGGGAGCTTGCCACCTACAGCGCCGGAG 650
                            **************************************************

MOX-1_MOX-F                 GCCTGCCACTGCAATTCCCCGAGGAGGTGGATTCATCCGAGAAGATGCGC 301
MOX-2_MOX-F                 GCCTGCCACTGCAATTCCCCGAGGAGGTGGATTCATCCGAGAAGATGCGC 300
gi|11761377|dbj|D13304.2|   GCCTGCCACTGCAATTCCCCGAGGAGGTGGATTCATCCGAGAAGATGCGC 700
                            **************************************************

MOX-1_MOX-F                 GCCTACTACCGCCAGTGGGCCCCTGTCTATTCGCCGGGCTCCCATCGCCA 351
MOX-2_MOX-F                 GCCTACTACCGCCAGTGGGCCCCTGTCTATTCGCCGGGCTCCCATCGCCA 350
gi|11761377|dbj|D13304.2|   GCCTACTACCGCCAGTGGGCCCCTGTCTATTCGCCGGGCTCCCATCGCCA 750
                            **************************************************

MOX-1_MOX-F                 GTACTCCAACCCCAGCATAGGGCTGTTCGGCCACCTGGCGGCGAGCAGCC 401
MOX-2_MOX-F                 GTACTCCAACCCCAGCATAGGGCTGTTCGGCCACCTGGCGGCGAGCAGCC 400
gi|11761377|dbj|D13304.2|   GTACTCCAACCCCAGCATAGGGCTGTTCGGCCACCTGGCGGCGAGCAGCC 800
                            **************************************************

MOX-1_MOX-F                 TGAAGCAGCCATTTGCCCAGTTGATGGAGCAGACCCTGCTGCCCGGGCTC 451
MOX-2_MOX-F                 TGAAGCAGCCATTTGCCCAGTTGATGGAGCAGACCCTGCTGCCCGGGCTC 450
gi|11761377|dbj|D13304.2|   TGAAGCAGCCATTTGCCCAGTTGATGGAGCAGACCCTGCTGCCCGGGCTC 850
                            **************************************************

MOX-1_MOX-F                 GGCATGCACCACACCT-TGTT------------------------------ 471
MOX-2_MOX-F                 GGCATGCACCACACCT-TGTCA----------------------------- 471
gi|11761377|dbj|D13304.2|   GGCATGCACCACACCTATGTCAATGTGCCGAAGCAGGCCATGGCGAGTTA 900
                            **************** ***
```

9.5.5 EBC Plasmid Gene

```
EBC-1_EBC-F                ----GCAA-CCCTGTTTGAGCTGGGCTCTATAAGTAAAACCTTCACCGGC 45
EBC-2_EBC-F                ---CGCAA-CCCTGTTTGAGCTGGGCTCTATAAGTAAAACCTTCACCGGC 46
gi|4558518|gb|M37839.2|    CCCCGCAAACCCTGTTTGAGCTGGGCTCTATAAGTAAAACCTTCACCGGC 1200
                               **** ****************************************

EBC-1_EBC-F                GTACTGGGCGGCGATGCCATTGCCCGGGGTGAAGTAGCGCTGGGCGATCC 95
EBC-2_EBC-F                GTACTGGGCGGCGATGCCATTGCCCGGGGTGAAGTAGCGCTGGGCGATCC 96
gi|4558518|gb|M37839.2|    GTACTGGGCGGCGATGCCATTGCCCGGGGTGAAATAGCGCTGGGCGATCC 1250
                           ******************************** ****************

EBC-1_EBC-F                GGTAGCAAAATACTGGCCTGAGCTCACGGGCAAGCAGTGGCAGGGCATTC 145
EBC-2_EBC-F                GGTAGCAAAATACTGGCCTGAGCTCACGGGCAAGCAGTGGCAGGGCATTC 146
gi|4558518|gb|M37839.2|    GGTAGCAAAATACTGGCCTGAGCTCACGGGCAAGCAGTGGCAGGGCATTC 1300
                           **************************************************

EBC-1_EBC-F                GCATGCTGGATCTGGCAACCTATACCGCAGGCGGTCTGCCGTTACAGGTG 195
EBC-2_EBC-F                GCATGCTGGATCTGGCAACCTATACCGCAGGCGGTCTGCCGTTACAGGTG 196
gi|4558518|gb|M37839.2|    GCATGCTGGATCTGGCAACCTATACCGCAGGCGGTCTGCCGTTACAGGTG 1350
                           **************************************************

EBC-1_EBC-F                CCGGATGAGGTCACGGATACCGCTTCTCTGCTGCGCTTTTATCAAAACTG 245
EBC-2_EBC-F                CCGGATGAGGTCACGGATACCGCTTCTCTGCTGCGCTTTTATCAAAACTG 246
gi|4558518|gb|M37839.2|    CCGGATGAGGTCACGGATACCGCCTCTCTGCTGCGCTTTTATCAAAACTG 1400
                           ********************** *************************

EBC-1_EBC-F                GCAGCCGCAGTGGAAG---------------------------------A 262
EBC-2_EBC-F                GCAGCCGCAGTGGAAG---------------------------------A 263
gi|4558518|gb|M37839.2|    GCAGCCGCAGTGGAAGCCGGGCACCACGCGTCTTTACGCTAACGCCAGCA 1450
                           ****************                                 *
```

9.6 ClustalW Analysis for the CTX-M Group 1 PCR Product

```
gi|39545937|gb|AY463958.1      ACTGCGCCAGTTCACGCTGATGGCGACGGCAACCGTCACGCTGTTGTTAG 250
EC-13441_ctxm1-f               ------------CACGC---------------------CGCTGTTGTTAG 17
                                           *****                     ************

gi|39545937|gb|AY463958.1      GAAGTGTGCCGCTGTATGCGCAAACGGCGGACGTACAGCAAAAACTTGCC 300
EC-13441_ctxm1-f               GA-GTGTGCCGCTGTATGCGCAAACGGCGGACGTACAGCAAAAACTTGCC 66
                               ** ********************************************

gi|39545937|gb|AY463958.1      GAATTAGAGCGGCAGTCGGGAGGCAGACTGGGTGTGGCATTGATTAACAC 350
EC-13441_ctxm1-f               GAATTAGAGCGGCAGTCGGGAGGCAGACTGGGTGTGGCATTGATTAACAC 116
                               **************************************************

gi|39545937|gb|AY463958.1      AGCAGATAATTCGCAAATACTTTATCGTGCTGATGAGCGCTTTGCGATGT 400
EC-13441_ctxm1-f               AGCAGATAATTCGCAAATACTTTATCGTGCTGATGAGCGCTTTGCGATGT 166
                               **************************************************

gi|39545937|gb|AY463958.1      GCAGCACCAGTAAAGTGATGGCCGCGGCCGCGGTGCTGAAGAAAAGTGAA 450
EC-13441_ctxm1-f               GCAGCACCAGTAAAGTGATGGCCGCGGCCGCGGTGCTGAAGAAAAGTGAA 216
                               **************************************************

gi|39545937|gb|AY463958.1      AGCGAACCGAATCTGTTAAATCAGCGAGTTGAGATCAAAAAATCTGACCT 500
EC-13441_ctxm1-f               AGCGAACCGAATCTGTTAAATCAGCGAGTTGAGATCAAAAAATCTGACCT 266
                               **************************************************

gi|39545937|gb|AY463958.1      TGTTAACTATAATCCGATTGCGGAAAAGCACGTCAATGGGACGATGTCAC 550
EC-13441_ctxm1-f               TGTTAACTATAATCCGATTGCGGAAAAGCACGTCAATGGGACGATGTCAC 316
                               **************************************************

gi|39545937|gb|AY463958.1      TGGCTGAGCTTAGCGCGGCCGCGCTACAGTACAGCGATAACGTGGCGATG 600
EC-13441_ctxm1-f               TGGCTGAGCTTAGCGCGGCCGCGCTACAGTACAGCGATAACGTGGCGATG 366
                               **************************************************

gi|39545937|gb|AY463958.1      AATAAGCTGATTGCTCACGTTGGCGGCCCGGCTAGCGTCACCGCGTTCGC 650
EC-13441_ctxm1-f               AATAAGCT---------------------------------------- 374
                               ********
```

9.7 ClustalW analysis following review of CIT primers

9.7.1 CIT Forward Primer

```
LAT-1           ATCCGGTCACGAAATACTGGCCAGAACTGACAGGCAAAAAGTGGCAGGGTATCCGCCTGC 520
CMY-2           ATCCGGTCACGAAATACTGGCCAGAACTGACAGGCAAACAGTGGCAGGGTATCCGCCTGC 2302
CMY-3_4_        ATCCGGTCACGAAATACTGGCCAGAACTGACAGGCAAACAGTGGCAGGGTATCCGCCTGC 379
CMY-4           ATCCGGTCACGAAATACTGGCCAGAACTGACAGGCAAACAGTGGCAGGGTATCCGCCTGC 379
CMY-5           ATCCGGTCACGAAATACTGGCCAGAACTGACAGGCAAACAGTGGCAGGGTATCCGCCTGC 2752
CMY-6           ATCCGGTCACGAAATACTGGCCAGAACTGACAGGCAAACAGTGGCAGGGTATCCGCCTGC 379
CMY-7           ATCCGGTCACGAAATACTGGCCAGAACTGACAGGCAAACAGTGGCAGGGTATCCGCCTGC 379
CMY-12          ATCCGGTCACGAAATACTGGCCAGAACTGACAGGCAAACAGTGGCAGGGTATCCGCCTGC 379
CMY-14          ATCCGGTCACGAAATACTGGCCAGAACTGACAGGCAAACAGTGGCAGGGTATCCGCCTGC 379
CMY-15          ATCCGGTCACGAAATACTGGCCAGAACTGACAGGCAAACAGTGGCAGGGTATCCGCCTGC 379
CMY-16          ATCCGGTCACGAAATACTGGCCAGAACTGACAGGCAAACAGTGGCAGGGTATCCGCCTGC 379
CMY-17          ATCCGGTCACGAAATACTGGCCAGAACTGACAGGCAAACAGTGGCAGGGTATCCGCCTGC 543
CMY-18          ATCCGGTCACGAAATACTGGCCAGAACTGACAGGCAAACAGTGGCAGGGTATCCGCCTGC 425
CMY-20          ATCCGGTCACGAAATACTGGCCCGAACTGACAGGCAAACAGTGGCAGGGTATCAGCCTGC 376
CMY-21          ATCCGGTCACGAAATACTGGCCAGAACTGACAGGCAAACAGTGGCAGGGTATCCGCCTGC 539
CMY-22          ATCCGGTCACGAAATACTGGCCAGAACTGACAGGCAAACAGTGGCAGGGTATCCGCCTGC 379
CMY-23          ATCCGGTCACGAAATACTGGCCAGAACTGACAGGCAAACAGTGGCAGGGTATCCGCCTGC 454
CMY-24          ATCCGGTCACGAAATACTGGCCAGAACTGACAGGCAAACAGTGGCAGGGTATCCGCCTGC 379
CMY-25          ATCCGGTCACGAAATACTGGCCAGAACTGACTGGCAAACAGTGGCAGGGTATCCGCCTGC 379
CMY-26          ATCCGGTCACGAAATACTGGCCAGAACTGACAGGCAAACAGTGGCAGGATATCAGCCTGC 379
CMY-27          ATCCGGTCACGAAATACTGGCCAGAACTGACAGGCAAACAGTGGCAGGGTATCCGCCTGC 379
CMY-28          ATCCGGTCACGAAATACTGGCCAGAACTGACAGGCAAACAGTGGCAGGGTATCCGCCTGC 379
CMY-29          ATCCGGTCACGAAATACTGGCCAGAACTGACAGGCAAACAGTGGCAGGGTATCCGCCTGC 379
CMY-30          ATCCGGTCACGAAATACTGGCCAGAACTGACAGGCAAACAGTGGCAGGGTATCCGCCTGC 379
CMY-31          ATCCGGTCACGAAATACTGGCCAGAACTGACAGGCAAACAGTGGCAGGGTATCCGCCTGC 379
CMY-32          ATCCGGTCACGAAATACTGGCCAGAACTGACAGGCAAACAGTGGCAGGGTATCCGCCTGC 379
CMY-33          ATCCGGTCACGAAATACTGGCCAGAACTGACAGGCAAACAGTGGCAGGGTATCCGCCTGC 379
CMY-34          ATCCGGTCACGAAATACTGGCCAGAACTGACAGGCAAACAGTGGCAGGGTATCAGCCTGC 379
CMY-35          ATCCGGTCACGAAATACTGGCCAGAACTGACAGGCAAACAGTGGCAGGGTATCCGCCTGC 379
CMY-36          ATCCGGTCACGAAATACTGGCCAGAACTGACAGGCAAACAGTGGCAGGGTATCCGCCTGC 2753
CMY-37          ATCCGGTCACGAAATACTGGTCAGAACTGACAGGCAAACAGTGGCAGGGTATCAGCCTGC 379
CMY-38          ATCCGGTCACGAAATACTGGCCAGAACTGACAGGCAAACAGTGGCAGGGTATCCGCCTGC 379
CMY-39          ATCCGGTCACGAAATACTGGCCAGAACTGACAGGCAAACAGTGGCAGGGTATCAGCCTGC 379
CMY-40          ATCCGGTCACGAAATACTGGCCAGAACTGACAGGCAAACAGTGGCAGGGTATCAGTCTGC 379
CMY-41          ATCCGGTCACGAAATACTGGCCAGAACTGACAGGCAAACAGTGGCGGGGTATCAGCCTGC 393
CMY-43          ATCCGGTCACGAAATACTGGCCAGAACTGACAGGCAAACAGTGGCAGGGTATCCGCCTGC 379
CMY-44          ATCCGGTCACGAAATACTGGCCAGAACTGACAGGCAAACAGTGGCAGGGTATCCGCCTGC 379
CMY-45          ATCCGGTCACGAAATACTGGCCAGAACTGACAGGCAAACAGTGGCAGGGTATCCGCCTGC 379
CMY-47          ATCCGGTCACGAAATACTGGCCAGAACTGACAGGCAAACAGTGGCGAGGTATCAGCCTGC 1418
CMY-48          ATCCGGTCACGAAATACTGGCCAGAACTGACAGGCAAACAGTGGCGGGGTATCAGCCTGC 1418
CMY-49          ATCCGGTCACGAAATACTGGCCAGAACTGACAGGCAAACAGTGGCAGGGTATCAGCCTGC 379
CMY-54          ATCCGGTCACGAAATACTGGCCAGAACTGACAGGCAAACAGTGGCAGGGTATCCGCCTGC 379
CMY-55          ATCCGGTCACGAAATACTGGCCAGAACTGACAGGCAAACAGTGGCAGGGTATCCGCCTGC 379
CMY-56          ATCCGGTCACGAAATACTGGCCAGAACTGACAGGCAAACAGTGGCAGGGTATCCGCCTGC 379
CMY-57          ATCCGGTCACGAAATACTGGCCAGAACTGACAGGCAAACAGTGGCAGGGTATCCGCCTGC 379
CMY-58          ATCCGGTCACGAAATACTGGCCAGAACTGACAGGCAAACAGTGGCAGGGTATCCGCCTGC 1646
CMY-59          ATCCGGTCACGAAATACTGGCCAGAACTGACAGGCAAACAGTGGCAGGGTATCCGCCTGC 421
CMY-64          ATCCGGTCACGAAATACTGGCCAGAACTGACAGGCAAACAGTGGCAGGGTATCAGCCTGC 379
CMY-73          ATCCGGTCACGAAATACTTGCCAGAACTGACAGGCAAGCAGTGGCAGGGTATCAGCCTGC 379
CFE-1           ATCCGGTCACGCAATACTGGCCTGAGCTGACGGGTAAGCAGTGGCAGGGTATCAGCCTGC 1386
                *********** ****** * * ** ***** ** **  ******  * **** * ****
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9.7.2 CIT Reverse Primer

```
LAT-1           CCAGCGTTATTGATATGGCCCGCTGGGTTCAGGCCAACATGGATGCCAGCCACGTTCAGG 934
CMY-2           CCAGCGTTATTGATATGGCCCGCTGGGTTCAGGCCAACATGGATGCCAGCCACGTTCAGG 2716
CMY-3_4_        CCAGCGTTATTGATATGGCCCGCTGGGTTCAGGCCAACATGGATGCCAGCCACGTTCAGG 793
CMY-4           CCAGCGTTATTGATATGGCCCGCTGGGTTCAGGCCAACATGGATGCCAGCCACGTTCAGG 793
CMY-5           CCAGCGTTATTGATATGGCCCGCTGGGTTCAGGCCAACATGGATGCCAGCCACGTTCAGG 3166
CMY-6           CCAGCGTTATTGATATGGCCCGCTGGGTTCAGGCCAACATGGATGCCAGCCACGTTCAGG 793
CMY-7           CCAGCGTTATTGATATGGCCCGCTGGGTTCAGGCCAACATGGATGCCAGCCACGTTCAGG 793
CMY-12          CCAGCGTTATTGATATGGCCCGCTGGGTTCAGGCCAACATGGATGCCAGCCACGTTCAGG 793
CMY-14          CCAGCGTTATTGATATGGCCCGCTGGGTTCAGGCCAACATGGATGCCAGCCACGTTCAGG 793
CMY-15          CCAGCGTTATTGATATGGCCCGCTGGGTTCAGGCCAACATGGATGCCAGCCACGTTCAGG 793
CMY-16          CCAGCGTTATTGATATGGCCCGCTGGGTTCAGGCCAACATGGATGCCAGCCACGTTCAGG 793
CMY-17          CCAGCGTTATTGATATGGCCCGCTGGGTTCAGGCCAACATGGATGCCAGCCACGTTCAGG 957
CMY-18          CCAGCGTTATTGATATGGCCCGCTGGGTTCAGGCCAACATGGATGCCAGCCACGTTCAGG 839
CMY-20          CCAGCGTTATTGATATGGCCCGCTGGGTTCAGGCCAACATGGATGCCAGCCACGTTCAGG 790
CMY-21          CCAGCGTTATTGATATGGCCCGCTGGGTTCAGGCCAACATGGATGCCAGCCACGTTCAGG 953
CMY-22          CCAGCGTTATTGATATGGCCCGCTGGGTTCAGGCCAACATGGATGCCAGCCACGTTCAGG 793
CMY-23          CCAGCGTTATTGATATGGCCCGCTGGGTTCAGGCCAACATGGATGCCAGCCACGTTCAGG 868
CMY-24          CCAGCGTTATTGATATGGCCCGCTGGGTTCAGGCCAACATGGATGCCAGCCACGTTCAGG 793
CMY-25          CCAGCGTTATTGATATGGCCCGCTGGGTTCAGGCCAACATGGATGCCAGCCACGTTCAGG 793
CMY-26          CCAACGTTACCGATATGGCACGCTGGGTTCAGGTCAACATGGACGCCAGCCGCGTTCAGG 793
CMY-27          CCAGCGTTATTGATATGGCCCGCTGGGTTCAGGCCAACATGGATGCCAGCCACGTTCAGG 793
CMY-28          CCAGCGTTATTGATATGGCCCGCTGGGTTCAGGCCAACATGGATGCCAGCCACGTTCAGG 793
CMY-29          CCAGCGTTATTGATATGGCCCGCTGGGTTCAGGCCAACATGGATGCCAGCCACGTTCAGG 793
CMY-30          CCAGCGTTATTGATATGGCCCGCTGGGTTCAGGCCAACATGGATGCCAGCCACGTTCAGG 793
CMY-31          CCAGCGTTATTGATATGGCCCGCTGGGTTCAGGCCAACATGGATGCCAGCCACGTTCAGG 793
CMY-32          CCAGCGTTATTGATATGGCCCGCTGGGTTCAGGCCAACATGGATGCCAGCCACGTTCAGG 793
CMY-33          CCAGCGTTATTGATATGGCCCGCTGGGTTCAGGCCAACATGGATGCCAGCCACGTTCAGG 793
CMY-34          CCAGCGTTATTGATATGGCCCGCTGGGTTCAGGCCAACATGGATGCCAGCCACGTTCAGG 793
CMY-35          CCAGTGTTATTGATATGGCCCGCTGGGTTCAGGTCAACATGGACGCCAGCCGCGTTCAGG 793
CMY-36          CCAGCGTTATTGATATGGCCCGCTGGGTTCAGGCCAACATGGATGCCAGCCACGTTCAGG 3167
CMY-37          CCAGCGTTATTGATATGGCACGCTGGGTTCAGGCCAACATGGATGCCAGCCGCGTTCAGG 793
CMY-38          CCAGCGTTATTGATATGGCCCGCTGGGTTCAGGCCAACATGGATGCCAGCCACGTTCAGG 793
CMY-39          CCAGCGTTATTGATATGGCCCGCTGGGTTCAGGTCAACATGGACGCCAGCCGCGTTCAGG 793
CMY-40          CCAGCGTTATCGATATGGCCCACTGGGTACAGGCCAACATGGACGCCAGCCACGTTCAGG 793
CMY-41          CCAGCGTTATCGATATGGCCCGCTGGGTTCAGGCCAACATGGACGCCAGCCACGTTCAGG 807
CMY-43          CCAGCGTTATTGATATGGCCCGCTGGGTTCAGGCCAACATGGATGCCAGCCACGTTCAGG 793
CMY-44          CCAGCGTTATTGATATGGCCCGCTGGGTTCAGGCCAACATGGATGCCAGCCACGTTCAGG 793
CMY-45          CCAGCGTTATTGATATGGCCCGCTGGGTTCAGGCCAACATGGATGCCAGCCACGTTCAGG 793
CMY-47          CCAGCGTTATCGATATGGCCCGCTGGGTTCAGGCCAACATGGACGCCAGCCACGTTCAGG 1832
CMY-48          CCAGCGTTATCGATATGGCCCGCTGGGTTCAGGCCAACATGGACGCCAGCCACGTTCAGG 1832
CMY-49          CCAACGTTACCGATATGGCCCGTTGGATTCAGGTCAACATGGACGCCAGCCGCGTTCAGG 793
CMY-54          CCAGCGTTATTGATATGGCCCGCTGGGTTCAGGCCAACATGGATGCCAGCCACGTTCAGG 799
CMY-55          CCAGCGTTATTGATATGGCCCGCTGGGTTCAGGCCAACATGGATGCCAGCCACGTTCAGG 793
CMY-56          CCAGCGTTATTGATATGGCCCGCTGGGTTCAGGCCAACATGGATGCCAGCCACGTTCAGG 793
CMY-57          CCAGCGTTATTGATATGGCCCGCTGGGTTCAGGCCAACATGGATGCCAGCCACGTTCAGG 793
CMY-58          CCAGCGTTATTGATATGGCCCGCTGGGTTCAGGCCAACATGGATGCCAGCCACGTTCAGG 2060
CMY-59          CCAGCGTTATTGATATGGCCCGCTGGGTTCAGGCCAACATGGATGCCAGCCACGTTCAGG 835
CMY-64          CCAGTGTTATTGATATGGCCCGCTGGGTTCAGGTCAACATGGACGCCAGCCGCGTTCAAG 793
CMY-73          CCAACGTTACCGATATGGCCCGTTGGATTCAGGTCAACATGGACGCCAGCCGCGTTCAGG 793
CFE-1           CAAGCGTTGTCGATATGACCCGCTGGGTCCAGGCCAACATGGATGCCAGCCAGGTTCAGG 1800
                * *  ***   ****** * *  *** * **** ********* ******* ***** *
```

9.8 ClustalW analysis for the review of ACC plasmid gene sequences

```
ACC-1          --CGATTAAAAGGTCACTTATTCATGCGTAAAA--AAATGCAGAA----CACAT--TGAA 666
ACC-2          --CGATTAAAAGGTCACTTATTCATGCGTAAAA--AAATGCAGAA----CACCT--TGAA 1593
ACC-3          ----------------------ATGCGTAAAA--AAATGCAGAA----CACCT--TGAA 29
ACC-4          CACGATTGAA--GCCACCGATCTGTTCCTTGAAGCAGGTGTATTATTTGCGCCTGGTAAA 1698
                                  * * *  **  *  ** *  *    * * *  * **

ACC-1          GCTGTTATCCGTGAT--TACC----TGTCTGG--CAGCAACTGTCCAAGGTGC------T 712
ACC-2          GCTGTTATCCGTGAT--TACC----TGTCTGG--CAGCAACTGCCCAAGGTGC------T 1639
ACC-3          GATGTTATCCGTGAT--TACC----TGTCTGG--CTTTAACGGCTCAGGGTGC------C 75
ACC-4          GCAGCGAACGCTGGTGGTGTCGCAACATCGGGGCTAGAAATGGCGCAGAACGCCGCTCGT 1758
               *  *  * *  ** *  *  *      ** **      **  *  **    **

ACC-1          CTGG-CTGCTAATATCGATGAGAGCAAAATTAAAG--ACACCG--------TTGATGACC 761
ACC-2          ATGG-CTGCCAATATCGATGAGAGCAAAATTAAAG--ACACCG--------TCGATGGCC 1688
ACC-3          ATGG-CGTCAGAAATGGATCAGGCCAAAATTAAAG--ACACCG--------TTGATAGCC 124
ACC-4          ATGGGCTG-GAAAGCCGA-GAAAGTGGATTTACGCCTACACCACATCATGCTGGATATCC 1816
                *** *     *    **  *      * ***     *****         * ***  **

ACC-1          TGATCCAGCC-GC--------TGATGCAGAAGAATAATATTCCCGGTATGTCGGTCGCAG 812
ACC-2          TAATCCAGCC-GC--------TGATGCAGAAGAATAATATTCCCGGTATGTCGGTCGCAG 1739
ACC-3          TGATCCAGCC-GC--------TGATGCAGAAGAATAATATTCCGGGCATGTCGGTGGCAG 175
ACC-4          --ACCAAGCCTGCGTGGAATATGGCGGAGAAGATAAGCAAACCCACTACGT---TCGCGG 1871
                 * * **** **        **  * ******  *  *  **    * **   * ** *

ACC-1          TG--ACCGTCAACGG------TAAAA--ACT-ACATT-TATAACTATGGGTTA-GCGGCA 859
ACC-2          TG--ACCATCAGAGG------TAGGA--ACT-ATATT-TATAACTACGGGTTA-GCGGCA 1786
ACC-3          TT--ACGCTGAACGG------TAAAA--ATT-ATATT-TATAACTATGGCTTA-GCCTCC 222
ACC-4          TGCAAACATCGCTGGATTTGTTAAAGTCGCTGATGCCATGCTGGCACAGGGCGTGCTGTA 1931
               *   *   *    **      **       * *     *      *  *     **

ACC-1          AAACAGCCTCAGCAGCCGGTTACGGAAAATA-CGTTATTTGAAGTGGG------------ 906
ACC-2          AAACAGCCTCAGCAGCCGGTGACGGAAAATA-CGTTATTTGAAGTGGG------------ 1833
ACC-3          AAACAGCCCCAGCAGCCCGTAACGGACAACA-CGCTATTTGAAGTTGG------------ 269
ACC-4          ATACC-CCTGAGAAATCGGTGACTCAACATATCGCCTCTCCAAACGAGAGGCGATATCTA 1990
               * **  **  ** *  * ** **  *  * * **    *  **    *

ACC-1          -----------------TTCGCTGAGT--AAAACGTT---TGCTGCCACC--TTGGCGTC 942
ACC-2          -----------------TTCGCTGAGT--AAAACGTT---TGCTGCCATC--TTGGCGTC 1869
ACC-3          -----------------CTCGCTGAGC--AAAACCTT---TGCAGCGACG--CTGGCGTC 305
ACC-4          TTTTCTACTTATTCCCTTCCAATGAGCTCAGGATTTTATACGCCACCGTCACTCGCTGAT 2050
                             *  ****   *  *  **    **  *        *  *

ACC-1          CTAT-GCGCAG---------GTGAGCGGTAAG----CTGTCTTT------GGATCAAA-G 981
ACC-2          CTAT-GCGCAG---------GCGAGCGGTAAG----CTGTCTTT------GGAGCAAA-G 1908
ACC-3          TTAT-GCACAG---------GTCAGCGGCAAG----TTATCGCT------GGATAAAA-G 344
ACC-4          CGATTGAGTAGTTTTTGTTAGCCAGCATCACGATCCCCATCTTCTTAGCAGGAACAAACG 2110
                 ** *   **         *  ***   * *       **         ***  *** *

ACC-1          CGTTA-----------GCCATTA--CGTTCCAGAGTTGCGTGGCAGCAGCTTT------- 1021
ACC-2          CGTTA-----------GCCACTA--TGTTCCAGAACTACGTGGCAGCAGCTTT------- 1948
ACC-3          CATTA-----------GCCATTA--TGTTCCAGAACTGCGCGGCAGCAGCTTC------- 384
ACC-4          CAATATAGGCACCGAAGCCGTTAGTTGATCCGGTCTTATTAATCCACACATTTTCCTGTG 2170
               *  **           ***  **   * *** *   *      *  **  **

ACC-1          --GACCACGTTAGCGTACTCAATGTGGGCACGCATACC-TCA--GGCCTACAGCTATTTA 1076
ACC-2          --GACCACGTTAGCGTACTCAATGTGGGTACGCATACC-TCA--GGTCTACAGCTGTTTA 2003
ACC-3          --GATCACATTAGCGTGCTGAATGCGGGAACGCATACC-ACA--GGTTTAGCGCTGTTCA 439
ACC-4          GCGGTAACGGCGGAACAATCGGCGTAGCCACGCTTTTCGTCATCGCCATATCGTTACCGG 2230
                 *   **    *     *    *  *  **** *  *  **  *   **  * *

ACC-1          TG-------CCGG--AAGATATTAAAAATA----------CCACA-CAG---CTGA-TGG 1112
ACC-2          TG-------CCGG--AAGATATCAAGAACA----------CCACA-CAG---CTGA-TGA 2039
ACC-3          TG-------CCTG--AAGAAGTGAAAAACA----------CCGAT-CAG---CTGA-TGG 475
ACC-4          TGAGCAAATTCGGCAGAGAAACCGGATATGGCAGCTGCTCCCACATCAGATCCTGAGTAA 2290
               **        * *   ***        *           **    ***   **** *

ACC-1          CTT------ATCTAAAA------GCATGGAAACCTG-------CCGATGCGGCT-GGAAC 1152
ACC-2          CTT------ATCTAAAA------GCATGGAAACCTG-------CTGATGCGGCT-GGAAC 2079
ACC-3          CTT------ATCTGAAA------GCGTGGAAACCCG-------CCGATCCTGCG-GGGAC 515
ACC-4          TCTCACCCGATTTGAAATAGCCGGTGTGGGTGGCTGTCAGAGCCTGTTGCATCTTGGCAT 2350
```

9.9 Excluded Isolates from the Regional Study

Table 9-2: Details of the 20 isolates excluded from the study on the basis of an incorrect species identification.

Study No.	Laboratory	Identification
DO-018	Dorchester	*Morganella morganii*
DO-043	Dorchester	*Enterobacter cloacae*
DO-049	Dorchester	*Serratia marcescens*
G-338	Gloucester	*Klebsiella pneumoniae*
G-343	Gloucester	*Stenotrophomonas maltophilia*
G-348	Gloucester	*Serratia fonticola*
G-349	Gloucester	*Serratia fonticola*
G-365	Gloucester	*Proteus mirabilis*
G-370	Gloucester	*Roultella ornitholytica*
G-372	Gloucester	*Enterobacter cloacae*
G-390	Gloucester	*Pseudomonas sp.*
G-391	Gloucester	*Klebsiella pneumoniae*
G-397	Gloucester	*Pseudomonas sp.*
G-401	Gloucester	*Citrobacter braakii*
G-409	Gloucester	*Morganella morganii*
G-411	Gloucester	*Morganella morganii*
G-420	Gloucester	*Citrobacter freundii*
G-442	Gloucester	*Citrobacter freundii*
TA-030	Taunton	*Enterobacter aerogenes*
TR-042	Truro	*Citrobacter freundii*

9.10 BLAST hit table for CMY-2 plasmid gene sequences

BLASTN 2.2.32+
Reference: Zheng Zhang, Scott Schwartz, Lukas Wagner, and Webb Miller (2000), "A greedy algorithm for aligning DNA sequences", J Comput Biol 2000; 7(1-2):203-14.

Reference for database indexing: Aleksandr Morgulis, George Coulouris, Yan Raytselis, Thomas L. Madden, Richa Agarwala, Alejandro A. Schaffer (2008), "Database Indexing for Production MegaBLAST Searches", Bioinformatics 24:1757-1764.

RID: V7FXPJU501R

Database: Nucleotide collection (nt)
31,279,139 sequences; 100,739,227,704 total letters
Query= Length=303

Sequences producing significant alignments:	Score (Bits)	E Value
gb|AB212086.1| Escherichia coli bla CMY-2 gene for beta-lacta...	560	5e-156
gb|KP037097.1| Escherichia coli strain NP2 CMY-2 beta-lactama...	560	5e-156
db|LC019731.1| Escherichia coli plasmid pCMY2 DNA, complete ...	560	5e-156
gb|KP340125.1| Salmonella enterica subsp. enterica SH6 CMY-2 ...	560	5e-156
gb|KP207590.1| Escherichia coli strain S62 plasmid pS62t stbE...	560	5e-156
gb|KP207588.1| Escherichia coli strain FS7E9S insertion seque...	560	5e-156
gb|KP207589.1| Escherichia coli strain S166 class C beta-lact...	560	5e-156
gb|KR494248.1| Escherichia coli strain M63 plasmid pESCR, com...	560	5e-156
db|AB672503.1| Salmonella enterica subsp. enterica serovar I...	560	5e-156
gb|KP789019.1| Escherichia coli strain WCHEC13-8 plasmid pCMY...	560	5e-156
emb|LN831184.1| Vibrio cholerae genome assembly V. cholerae 1...	560	5e-156
gb|KP742988.1| Salmonella enterica subsp. enterica serovar Se...	560	5e-156
gb|CP011429.1| Salmonella enterica subsp. enterica strain YU3...	560	5e-156
gb|KP281294.1| Escherichia coli strain 1152602 class C beta-l...	560	5e-156
gb|KJ802405.1| Providencia stuartii isolate GN576 plasmid pND...	560	5e-156
gb|KJ802404.1| Escherichia coli isolate GN568 plasmid pNDM-Ec...	560	5e-156
gb|CP009413.2| Salmonella enterica strain CFSAN007427 plasmid...	560	5e-156
gb|CP009412.2| Salmonella enterica strain CFSAN007426 plasmid...	560	5e-156
gb|CP009411.2| Salmonella enterica strain CFSAN007425 plasmid...	560	5e-156
gb|CP009410.2| Salmonella enterica strain CFSAN007405 plasmid...	560	5e-156
gb|CP009409.2| Salmonella enterica subsp. enterica serovar He...	560	5e-156
gb|KP205272.1| Escherichia coli strain EL-231 plasmid pQEL231...	560	5e-156
dbj|AB906452.1| Salmonella enterica subsp. enterica serovar I...	560	5e-156
dbj|AB750370.1| Salmonella enterica subsp. enterica serovar I...	560	5e-156
gb|KJ588779.1| Klebsiella pneumoniae strain ATCC BAA-2146 pla...	560	5e-156
gb|KM507172.1| Escherichia coli strain CNR581 CMY-121 beta-la...	560	5e-156
gb|KP017243.1| Klebsiella pneumoniae strain Kp2735 plasmid p2...	560	5e-156
gb|KP056256.1| Escherichia coli strain YDC637 plasmid pYDC637...	560	5e-156
gb|CP009564.1| Salmonella enterica subsp. enterica serovar Ne...	560	5e-156
gb|CP009562.1| Salmonella enterica subsp. enterica serovar Ne...	560	5e-156
gb|CP009560.1| Salmonella enterica subsp. enterica serovar Ne...	560	5e-156
gb|CP009570.1| Salmonella enterica subsp. enterica serovar Ne...	560	5e-156
gb|CP009567.1| Salmonella enterica subsp. enterica serovar Ne...	560	5e-156
gb|CP009566.1| Salmonella enterica subsp. enterica serovar Ne...	560	5e-156
gb|CP007636.1| Vibrio cholerae strain 2012EL-2176 plasmid, co...	560	5e-156
gb|KJ909290.1| Aeromonas salmonicida subsp. salmonicida strai...	560	5e-156
gb|KC585021.1| Escherichia coli strain E34OD insertion sequen...	560	5e-156
emb|HG970648.1| Escherichia coli plasmid pJIE512b, complete s...	560	5e-156
dbj|AP014565.1| Salmonella enterica subsp. enterica serovar T...	560	5e-156
emb|HG941718.1| Escherichia coli ST131 strain EC958 chromosom...	560	5e-156
dbj|AB933352.1| Klebsiella pneumoniae DNA, insertion sequence...	560	5e-156
dbj|AB933351.1| Klebsiella pneumoniae DNA, insertion sequence...	560	5e-156
dbj|AB933350.1| Klebsiella pneumoniae DNA, insertion sequence...	560	5e-156
gb|KJ496347.1| Escherichia coli strain C1310573 beta-lactamas...	560	5e-156
gb|KJ155695.1| Serratia marcescens strain A4Y201 class C beta...	560	5e-156
gb|KJ488945.1| Escherichia coli strain C1050854 CMY-2 beta-la...	560	5e-156
gb|KJ135996.1| Escherichia coli strain 4-462 class C beta-lac...	560	5e-156
gb|KJ135995.1| Escherichia coli strain 4-67 class C beta-lact...	560	5e-156
gb|KJ135971.1| Escherichia coli strain 1-46 class C beta-lact...	560	5e-156

9.11 BLAST hit table for DHA-1 plasmid gene sequences

BLASTN 2.2.32+
Reference: Zheng Zhang, Scott Schwartz, Lukas Wagner, and Webb Miller (2000), "A greedy algorithm for aligning DNA sequences", J Comput Biol 2000; 7(1-2):203-14.

Reference for database indexing: Aleksandr Morgulis, George Coulouris, Yan Raytselis, Thomas L. Madden, Richa Agarwala, Alejandro A. Schaffer (2008), "Database Indexing for Production MegaBLAST Searches", Bioinformatics 24:1757-1764.

RID: V7HW61ET01R

Database: Nucleotide collection (nt)
31,279,139 sequences; 100,739,227,704 total letters
Query= Length=359

Sequences producing significant alignments:	Score (Bits)	E Value
gb|KP683353.1| Escherichia coli strain DHA-1 AmpC (ampC) gene...	664	0.0
gb|AY635140.1| Klebsiella pneumoniae beta-lactamase DHA-1 gen...	664	0.0
emb|LN831185.1| Vibrio cholerae genome assembly V. cholerae 1...	654	0.0
dbj|LC055503.1| Klebsiella pneumoniae plasmid pHM881QN DNA, c...	654	0.0
gb|KJ187752.1| Klebsiella pneumoniae strain 7433 plasmid pTR2...	654	0.0
gb|KF856624.1| Proteus mirabilis strain PEL ThdF (thdF) gene,...	654	0.0
gb|KM087850.1| Morganella morganii class C beta-lactamase (bl...	654	0.0
gb|KM111265.1| Escherichia coli strain NF27595(CI13) insertio...	654	0.0
emb|HG918041.1| Klebsiella pneumoniae Kp15 plasmid pENVA comp...	654	0.0
gb|KJ504228.1| Klebsiella pneumoniae strain KUN-8056 plasmid ...	654	0.0
gb|KJ135992.1| Acinetobacter baumannii strain 4-495 class C b...	654	0.0
gb|KJ135991.1| Klebsiella pneumoniae strain 4-448 class C bet...	654	0.0
gb|KJ135990.1| Klebsiella pneumoniae strain 4-12 class C beta...	654	0.0
gb|KJ135977.1| Klebsiella pneumoniae strain 2-66 class C beta...	654	0.0
gb|KJ135976.1| Klebsiella pneumoniae strain 2-50 class C beta...	654	0.0
gb|KJ135975.1| Klebsiella pneumoniae strain 2-34 class C beta...	654	0.0
gb|KJ135973.1| Klebsiella pneumoniae strain 2-12 class C beta...	654	0.0
gb|KJ135972.1| Enterobacter cloacae strain 2-8 class C beta-l...	654	0.0
gb|KJ135967.1| Klebsiella pneumoniae strain 1-47 class C beta...	654	0.0
gb|KJ135966.1| Klebsiella pneumoniae strain 1-39 class C beta...	654	0.0
gb|KJ135965.1| Klebsiella pneumoniae strain 1-28 class C beta...	654	0.0
gb|KJ127282.1| Klebsiella pneumoniae strain T9 plasmid class ...	654	0.0
gb|KJ127281.1| Klebsiella pneumoniae strain F63 plasmid class...	654	0.0
gb|KJ127279.1| Klebsiella pneumoniae strain F58 plasmid class...	654	0.0
gb|KJ127276.1| Klebsiella pneumoniae strain F52 plasmid class...	654	0.0
gb|KJ127275.1| Klebsiella pneumoniae strain F50 plasmid class...	654	0.0
gb|KJ127274.1| Klebsiella pneumoniae strain F48 plasmid class...	654	0.0
gb|KJ127273.1| Klebsiella pneumoniae strain F44 plasmid class...	654	0.0
gb|KJ127272.1| Klebsiella pneumoniae strain F42 plasmid class...	654	0.0
gb|KJ127271.1| Klebsiella pneumoniae strain F33 plasmid class...	654	0.0
gb|KJ127270.1| Klebsiella pneumoniae strain F31 plasmid class...	654	0.0
gb|KJ127261.1| Klebsiella pneumoniae strain K40 plasmid class...	654	0.0
gb|KJ127260.1| Klebsiella pneumoniae strain K39 plasmid class...	654	0.0
gb|KJ127251.1| Klebsiella pneumoniae strain F26 plasmid class...	654	0.0
gb|KJ127250.1| Klebsiella pneumoniae strain F19 plasmid class...	654	0.0
gb|KJ127249.1| Klebsiella pneumoniae strain F16 plasmid class...	654	0.0
gb|KJ127248.1| Klebsiella pneumoniae strain F9 plasmid class ...	654	0.0
gb|KF793937.1| Klebsiella pneumoniae plasmid pKPS30, complete...	654	0.0
gb|KF646592.1| Enterobacter hormaechei subsp. oharae strain C...	654	0.0
gb|KF250428.1| Klebsiella pneumoniae strain CRE114 plasmid pI...	654	0.0
ref|NG_041672.1| Klebsiella pneumoniae plasmid pRI 1 QnrB38 (...	654	0.0
gb|KC999035.1| Escherichia coli plasmid pEC2-NDM-3, partial s...	654	0.0
gb|KC848778.1| Escherichia coli strain DEC216 plasmid pDEC216...	654	0.0
dbj|AB759690.1| Klebsiella pneumoniae plasmid pNDM-1saitama01...	654	0.0
gb|CP004345.1| Morganella morganii subsp. morganii KT, comple...	654	0.0
gb|JQ356870.1| Citrobacter freundii strain Iona 6 plasmid pCF...	654	0.0
gb|JN215524.1| Citrobacter freundii strain Iona 4 plasmid pCF...	654	0.0
gb|JN215523.1| Citrobacter freundii strain Iona 2 plasmid pCF...	654	0.0
gb|JX988621.1| Klebsiella pneumoniae strain 601 plasmid pNDM-...	654	0.0
gb|JX495964.1| Escherichia coli strain 11-905 beta-lactamase ...	654	0.0
gb|JX268774.1| Klebsiella pneumoniae strain AKH11/C53 beta-la...	654	0.0

9.12 Informal Audit of ESBL Reporting Rates, 2013.

In 2013, as part of the South West Regional Biomedical Group (a quarterly forum of microbiology scientific staff), a small audit was undertaken of basic ESBL reporting rates in laboratories in the South West. Five laboratories were able to provide data for 2012 for urine samples and seven laboratories provided data for blood culture samples. The purpose of this informal audit was to identify whether there were variations in the ESBL-positivity rates between laboratories. Reported ESBL positivity rates varied from 1.3% to 6.2% for urine isolates and from 0.4% to 14.8% for blood culture isolates (**Figure 9-1**). The laboratories tested a total of 64,017 blood culture samples and 320,775 urine samples during 2012. From these samples, 46,260 isolates of *E. coli* were isolated, most of which were from urine samples (97%). A positive ESBL confirmation result was reported in a total of 1968, giving an overall prevalence rate of 4.3% for the presence of ESBL enzymes. The rate was significantly higher in the blood culture samples at 5.8%, compared to 4.2% for urine samples ($p=0.003$, χ^2). Although only an informal audit, the study did provide some evidence of variation in ESBL prevalence rates between laboratories in the South West region. The results of this audit were presented to the SWRB Group on 4th June 2013 by the author of the current study (Lewis, 2013).

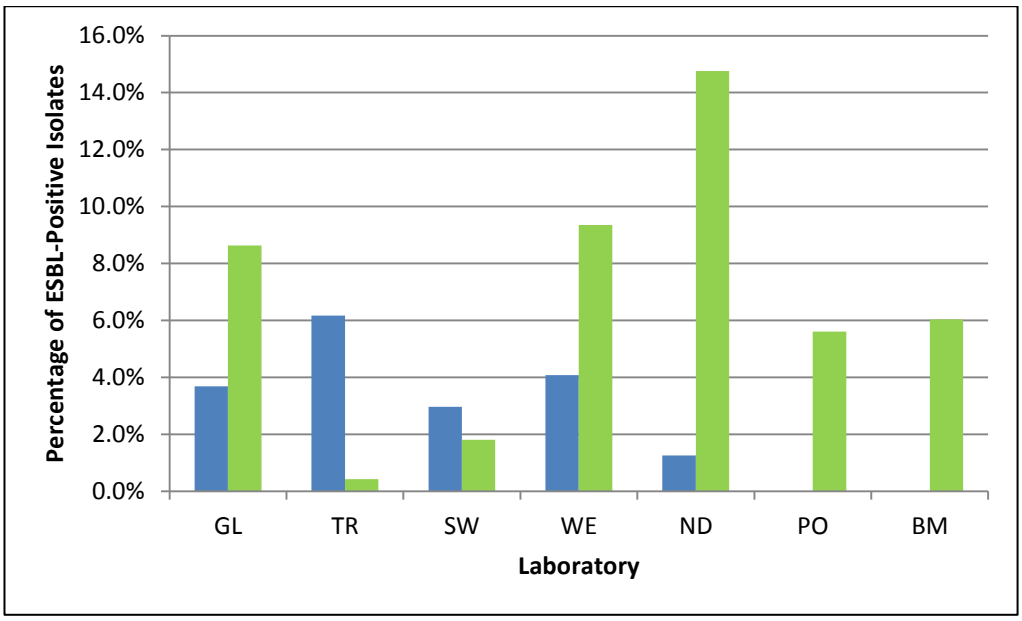

Figure 9-1: ESBL positivity rates between laboratories for urine samples (blue) and blood cultures (green); identified as part of an informal audit of five laboratories in the South West region.

Data were obtained from a regional audit undertaken in 2013 (Lewis, 2013). The presence of ESBL enzymes was determined using the phenotypic methods utilised by the various laboratories.

Lab Key: Gloucester (GL), Truro (TR), Swindon (SW), Weston-Super-Mare (WE), North Devon (ND), Poole (PO) and Bournemouth (BM).

9.13 Copy of Published Material – Lewis *et al.* (2015)

This is the authors' accepted manuscript of an article published as the version of record in the British Journal of Biomedical Science (January 2015), available at: http://www.tandfonline.com/doi/abs/10.1080/09674845.2015.11666789

Chromosomal *ampC* Mutations in Cefpodoxime-Resistant ESBL-Negative Uropathogenic *Escherichia coli*

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Abstract

AmpC beta-lactamase is an enzyme commonly produced by *E. coli* that causes resistance to cephalosporins and penicillins. Enzyme production is controlled by the strength of the promoter encoded by the chromosomal *ampC* gene, with the level of production affected by the presence of certain mutations in this region. This study set out to determine the prevalence of *ampC* promoter mutations present in a group of uropathogenic *E. coli* strains.

A total of 50 clinical strains of *E. coli* were collected from urine samples between June 2011 and November 2011. Strains were investigated for the presence of mutations in the chromosomal *ampC* promoter region by amplification and sequencing of a 271bp product. The presence of *ampC*-carrying plasmids derived from other species was also determined, to exclude these from further analysis.

ampC-carrying plasmids were found in 10 of the 50 strains, all of which were of the CIT-type. Analysis of the chromosomal *ampC* promoter region in the 40 remaining strains showed mutations at 16 different positions, with 18 different genotype patterns detected overall. The most common *ampC* chromosomal mutation, present in 25 of 40 strains, was a T→A transition at position -32. This mutation has been shown by others to increase enzyme production by up to 46-fold.

Altogether, three separate mutations (-32, -42 and -13ins) were present in 90% of the 40 non-plasmid strains, indicating a strong association with the resistance observed. It appears, therefore, that the majority of AmpC-mediated resistance in *E. coli* can be accounted for by just three point mutations in the chromosome.

Keywords

Antibiotic resistance

Cephalosporin

Escherichia coli

AmpC beta-lactamase

Introduction

AmpC beta-lactamase is an enzyme commonly produced by *Escherichia coli* that causes resistance to cephalosporins and penicillins.[1] All strains of *E. coli* carry the chromosomal *ampC* gene for enzyme production, which is normally weakly expressed and under tight control by regulatory mechanisms.[2] Production of the chromosomally-encoded enzyme is constitutive, but at such a low level that clinical failure of beta-lactam antibiotics is not usually seen. Certain mutations can occur in the promoter region, affecting the level of enzyme production. Strains with chromosomal *ampC* mutations can produce enzymes in higher amounts and are said to hyper-produce the AmpC enzyme, leading to clinical resistance and treatment failures.[3]

The common DNA sequence seen in numerous *E. coli* promoters is a -35 box

(TTGACA) separated from a -10 box (TATAAT) by 17bp.[2] The normal *ampC* promoter sequence, however, includes single nucleotide difference in each of these hexameric boxes, together with a spacer difference of 16bp (Figure 1). These small differences are sufficient to affect the function of the promoter and decrease the normal AmpC enzyme production to its constitutive low level.[4]

Mutations in the *ampC* promoter region can arise, and include transitions and insertions in the -35 or -10 boxes, which create a region more closely related to the standard *E. coli* promoter sequence, and thus a stronger promoter.[2] The most frequently reported promoter mutation (C→T at position -42) is one that creates a displaced -35 box in the promoter sequence, and is associated with a 20-fold increase in enzyme production.[5] Other reported key mutations include substitutions that change the sequence of the wild-type -35 box itself, and insertions in the spacer region between the -35 and -10 boxes. Mutations have also been reported throughout other locations in the promoter, attenuator and coding regions, but these are considered to have a lesser impact on the level of enzyme production.[5]

In addition to chromosomal *ampC* mutations, *E. coli* can also acquire the genes for AmpC enzyme production from other species. First described in 1988, plasmid *ampC* genes are derived from species such as *Enterobacter* spp. and *Citrobacter* spp..[6] Although there are >200 different plasmids reported to carry *ampC* genes, for convenience they are usually classified into six groups based on the species of origin; CIT, ACC, DHA, FOX, MOX, and EBC.[7] The CMY-2 plasmid (within the CIT group) is the most common AmpC plasmid encountered to date, and also has the largest geographic spread.[1] Woodford *et al*. (2007)[8] tested 135 strains of *E. coli* referred from UK laboratories for the investigation of unusual resistance patterns, detecting an *ampC*-carrying plasmid in 49%. The majority were determined to be of the CIT-group, but ACC, FOX and DHA groups were also detected. Strains with plasmids carrying *ampC* genes were found to be more resistant to third-generation cephalosporins than those with *ampC* chromosomal promoter mutations.

This study set out to characterise the chromosomal *ampC* mutations present in a group of uropathogenic *E. coli* strains. Although *E. coli* is one of the most common pathogens isolated in clinical laboratories, there is a lack of data for the UK describing the prevalence and nature of AmpC resistance in clinical isolates. This is particularly the case for the chromosomal mutations responsible for AmpC enzyme hyper-production. Whilst the *ampC*-carrying plasmids can give rise to a higher level of resistance, the chromosomal *ampC* mutations seen in *E. coli* represent a larger overall group of resistant strains.

Materials & Methods

Strain Collection

Clinical strains of *E. coli* isolated from urine samples in the Gloucestershire laboratory between June 2011 and November 2011 were included if disc susceptibility testing indicated cefpodoxime resistance with a subsequent negative result for clavulanic acid synergy; thus excluding the presence of ESBL-mediated resistance.[9] Strains were identified to species level using a chromogenic urine media plate (257481, Becton Dickinson, Oxford, UK) and API20E identification strips

(20100, Biomerieux, Basingstoke, UK). Isolates were excluded if the same species had previously been isolated from the patient within a 28-day period. Strains were anonymised before inclusion in the study, and only basic patient demographic data (e.g. age and gender) were collected for each sample. During the collection period, a total of 50 clinical urine strains were included.

Susceptibility Testing

Strains were tested for susceptibility to a range of cephalosporins, including the antibiotics cefpodoxime (10µg), cefuroxime (30µg), cefoxitin (30µg), cefotaxime (30µg) and cefepime (30µg), using a standardised disc susceptibility method.[10] A 0.5 MacFarland suspension was prepared and diluted to a 1:100 concentration. The final suspension was inoculated onto an Isosensitest agar plate (PO0779A, Oxoid, Basingstoke, UK) using a cotton-tipped swab. Antibiotic discs (various, Oxoid, Basingstoke, UK) were applied to the surface of the agar and the plate was incubated for 18-24 hours at 37^{o}C in air. Following incubation, the zone size for each antibiotic disc was recorded.

Strains were also tested to determine the minimum inhibitory concentration (MIC) of cefotaxime. MICE gradient strips (MA0111F, Oxoid, Basingstoke, UK) were used to test the cefotaxime MIC within the range 0.002 to 32mg/L. A 0.5 MacFarland suspension was prepared and inoculated directly onto an Isosensitest agar plate using a cotton-tipped swab. The MICE strip was applied to the surface of the agar and the plate was incubated for 18-24 hours at 37^{o}C in air. Following incubation, the point of intersection of the zone to the strip was recorded as the MIC for the strain.

Detection of ampC Plasmids

DNA templates for PCR amplification were prepared using a crude-lysis method.[11] Strains were incubated overnight on Columbia Horse Blood agar plates (PB0122A, Oxoid, Basingstoke, UK). A heavy bacterial suspension, equivalent to MacFarland standard 4.0, was prepared in 100µl water. Tubes were vortex-mixed for 2 minutes and then centrifuged for 5 minutes at 8,000g. The resulting supernatants were used as the DNA template.

PCR assays were run on the SmartCycler II instrument (Cepheid, Sunnyvale, US), using the DX software (Version 3.0). Assay parameters were those recommended for use with the Quantifast SYBR Green master-mix (204054, Qiagen, Manchester, UK): 95^{o}C for 5 minutes, followed by 35 cycles of 95^{o}C for 10 seconds and 60^{o}C for 30 seconds. Fluorescence was read after each cycle at the instrument settings for FAM dye. A melting curve protocol was run at the end of amplification, with the temperature increasing from 60^{o}C to 95^{o}C at a rate of 0.5^{o}C / sec. The resulting dissociation curve was used to visualise the presence of an amplified product.
The presence of AmpC plasmid groups was determined using two multiplex real-time SYBR Green PCR assays (CIT/ACC/DHA and FOX/MOX/EBC). Primers for five of the plasmid groups were as previously described.[7] The CIT primers were updated to include more recently reported plasmids: CIT-F (5'-TGA TGC AGG AGC AGG CTA TTC-3') and CIT-R (5'-ACA GAC CAA TGC TGG AGT TAG-3'). Primers (Invitrogen, Paisley, UK) were used at a 0.2µM final concentration. Multiplex AmpC plasmid assays with positive dissociation curves were confirmed using the same primers in three separate simplex reactions, to identify the individual plasmid present.

Sequencing the ampC Promoter Region

A 271bp region of the *ampC* gene, including the promoter region, attenuator region and part of the coding region, was amplified for each isolate using previously published primers at 0.1µM concentration.[3] PCR protocol parameters were: 95°C for 5 minutes, followed by 35 cycles of 95°C for 10 seconds and 60°C for 30 seconds. A melting curve protocol was run to confirm the presence of an amplified product prior to sequencing. PCR products were sent to an external company (Eurofins Genomics, Ebersberg, Germany) for purification and sequencing, using the forward amplification primer. The reported sequence of each product was compared against the GenBank entry for *E. coli* NCTC 12241 (GenBank Accession AY899338), a laboratory control strain. Sequences were aligned using the ClustalW online software (www.ebi.ac.uk/tools/msa/clustalw2). The nature and position of each mutation was noted, and recorded according to the numbering system of Jaurin *et al*. (1981).[12]

Results

The 50 collected strains comprised 75% from female patients, and 25% from male. The mean age of patients was 58.3 years, with a range from 1 to 94 years. The results for disc susceptibility testing are shown in Table 1. The majority (98%) of isolates were resistant to cefuroxime when zone sizes were compared against breakpoints set by the British Society of Antimicrobial Chemotherapy (BSAC).[10] In contrast, only 28% of strains demonstrated resistance to cefotaxime. Results also showed 98% of strains were resistant to cefoxitin, and there were 22 strains (44%) with resistant or intermediate zone sizes to the fourth-generation cephalosporin cefepime.

Ten strains demonstrated an amplification product typical of the CIT group of plasmids. None of the other plasmid groups (ACC, DHA, FOX, MOX, EBC) were detected. As the genes carried on AmpC plasmids can account for enzyme production alone, these strains were excluded from further analysis.

From the remaining 40 strains, sequencing the 271bp region covering the chromosomal *ampC* promoter showed mutations at 16 different positions throughout the amplified region (See Table 2). Overall, 18 different genotype patterns were detected from the 40 strains investigated, with all strains containing at least one polymorphism when compared to the sequence for the *E. coli* control strain. The most common chromosomal *ampC* genotype (n=11) included a T→A substitution at position -32, together with substitutions at positions +58 and +63. The -32 mutation alters the wild-type -35 box from TTGTCA to TTGACA, one that is closer to the *E. coli* standard promoter sequence.[5] The altered -35 box retains its original position in the promoter region. Mutations related to a displaced promoter region were found in three strains, at positions -42 and -18. The -42 (C→T) substitution creates a new -35 box, whereas the G→A mutation at -18 gives rise to a new -10 box region. These mutations are often reported together,[13] and result in two new regions 17bp apart at different locations. The increased distance between the two regions has been reported to affect promoter strength.[5] The -18 mutation was also observed in two strains without the -42 mutation present.

Insertions in the spacer region (position -13) were detected in eight strains, increasing the distance between the -35 and -10 boxes from 16bp through the insertion of 1 or 2 base pairs. Seven strains had mutations in the attenuator region (positions +17, +23 and +37), often present along with promoter mutations. A total of 19 strains were found to have mutations in the coding region. The three mutations at positions +63, +70 and +81 have been reported to alter the amino acid coded for at each of the three respective codons. However, it is not clear whether this has a significant impact on enzyme function.[13]

The geometric mean MIC for cefotaxime was 1.2mg/L for all strains, just above the recommended breakpoint of 1.0mg/L.[10] Those strains with the -32 mutation present had a lower cefotaxime MIC of 0.6mg/L, compared to 1.2mg/L for strains with other mutations. In contrast, the ten strains with a CIT plasmid present had a geometric mean cefotaxime MIC of 7.3mg/L.

Discussion

CIT-type plasmids were found in 20% of the 50 strains collected. Although not fully characterised in this study, these are usually reported to be the CMY-2 plasmid. The presence of CMY-2 plasmids has been reported globally in clinical samples, and has also been associated with foodstuffs and animals.[14–16] Phylogenetic studies have shown that *E. coli* strains carrying AmpC plasmids are more likely to be virulent, pathogenic types, whereas the strains with chromosomal promoter mutations are more likely to be commensal or environmental strains.[17]

A total of 28 (70%) of the remaining 40 strains possessed either the -42 or -32 mutation, both considered to be key factors in creating a stronger *ampC* promoter by creating a -35 box with greater homology to the *E. coli* standard promoter sequence.[5] The -32 mutation was predominant in this study, with 25 strains demonstrating this substitution, either alone or in conjunction with other mutations. This mutation has been reported to result in an 8- to 46-fold increase in over-expression when compared to wild type strains.[5] In this study, the geometric mean MIC for cefotaxime in the -32 mutation group was 0.6mg/L, compared to 0.06mg/L for the *E. coli* control strain (NCTC 12241), showing a ten-fold increase.

The -42 mutation and -13 spacer insertions have been shown to effect a similar impact on the level of over-expression; 20-fold and 24- to 61-fold, respectively.[5] Whilst we found only three strains with the -42 mutation, other studies have reported a higher prevalence,[13,17–19] with one study finding 100% of isolates with this mutation.[20] Eight of the 40 strains in our study had insertions of either one or two bases at position -13. The inserted bases were either adenine or thymine, and increased the spacer region from 16 base pairs to 17 or 18 base pairs. Seven strains were found to have mutations in the attenuator region. Although attenuator region mutations are thought to increase enzyme production through the destabilisation of the stem-loop structure, Tracz *et al*. (2007) demonstrated that these mutations have little actual effect on the level of enzyme production.[5]

Resistance to cefoxitin is proposed as a screening test for AmpC production.[10] In this study, 98% of all strains were resistant by disc susceptibility testing, confirming it's utility as such. Whilst this may represent a good method for detecting AmpC-

mediated resistance, the specificity of the method is reduced by other means in which strains can become resistant to cefoxitin (e.g. membrane permeability).[21] AmpC-producing strains are generally considered to have the antibiogram phenotype of cefoxitin-resistant, cefepime-sensitive.[8] Here, only 56% of strains met the criteria for both, with only 42% having a zone size above the breakpoint of 32mm for cefepime susceptibility.

In this study of 50 uropathogenic strains of cefpodoxime-resistant, ESBL-negative *E. coli*, AmpC beta-lactamase resistance was confirmed in 92% of isolates. Although some strains did carry a plasmid *ampC* gene, the majority of strains possessed one of the *ampC* chromosomal promoter region mutations recognised to cause enzyme hyper-production. The -32 mutation, -42 mutation and -13 insertions accounted for 90% of the resistance in the 40 non-plasmid strains, but were not found together in the same strain. Thus, providing further evidence that these represent the key mutations responsible for enzyme hyper-production. It was of interest to note a predominance of the -32 mutation in the strains, rather than the -42 mutation reported elsewhere. Although unlikely to represent a difference in the level of clinical resistance, strains carrying the -32 mutation may represent a dominant clone in the local population. Further studies are underway to include strains from other laboratories and to utilise molecular typing methods to identify the presence of different resistant strain populations.

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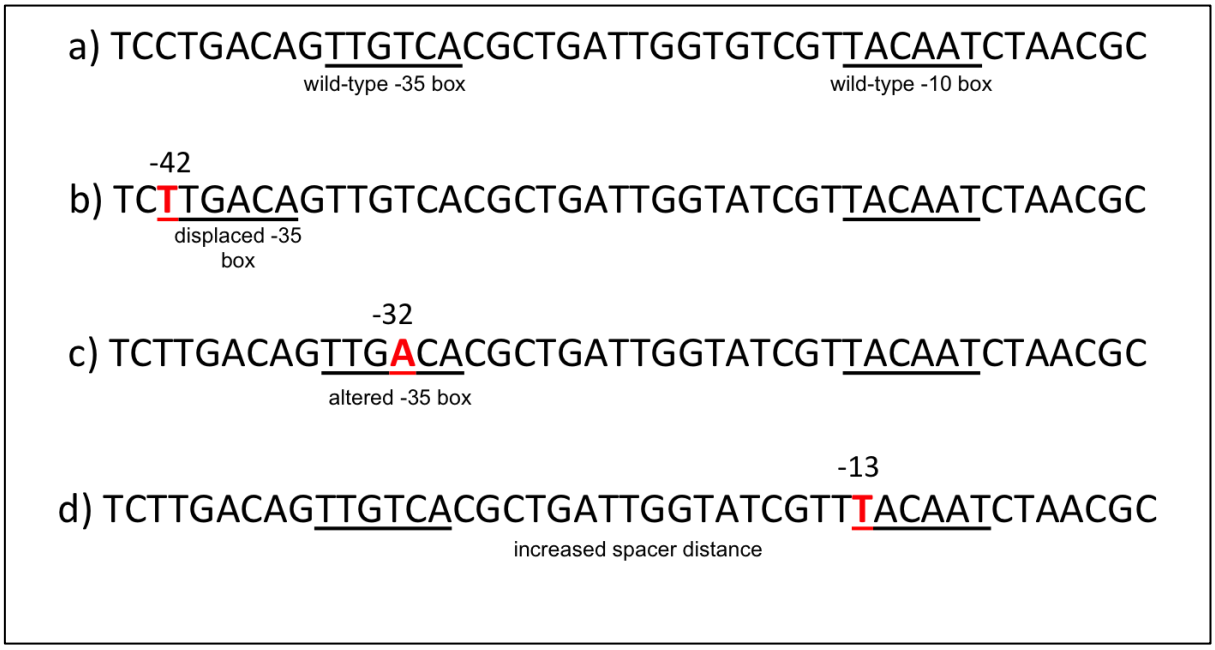

Figure 1: Section of *ampC* promoter region, showing the positions of:

a) The wild-type hexameric -10 and -35 boxes
b) C->T transition at position -42 creates a displaced -35 box with a sequence identical to the *E. coli* standard promoter (TTGACA)
c) T->A transition at position -32 creates an altered wild-type -35 box with a sequence identical to the *E. coli* standard promoter (TTGACA)
d) Insertion of a single nucleotide at position -13 increases the spacer distance from 16bp to 17bp

Numbering of positions is according to Jaurin *et al*. (1981)[12]

	Disc Content (µg)	Zone Diameter Breakpoints (mm)	S (%)	I (%)	R (%)
Cefpodoxime	10	≤19	0 (0)	N/A	50 (100)
Cefuroxime	30	≤19	1 (2)	N/A	49 (98)
Cefotaxime	30	≤23 to ≤29	14 (28)	22 (44)	14 (28)
Cefoxitin	30	≤22	1 (2)	N/A	49 (98)
Cefepime	30	≤26 to ≤31	28 (56)	12 (24)	10 (20)

Table 1: Disc susceptibility results for the 50 strains to a range of five cephalosporins. The zone diameter breakpoints shown are for resistant strains, or for resistant and intermediate strains if a range is given.

S = Resistant, I = Intermediate, R = Resistant

Genotype	No. of Strains	-42	-32	-28	-18	-14	-13	-13ins	-1	+6	+17	+23	+34	+37	+58	+63	+70	+81
Control		C	T	G	G	T	T	-	C	C	C	G	G	G	C	T	C	A
G1	11		A												T	C		
G2	8		A															
G3	2		A														T	G
G4	2							T		T								
G5	2	T			A				T						T			G
G6	2			A				AT										
G7	2			A		A		T			T							
G8	1				A				T						T			G
G9	1			A														
G10	1			A				T										
G11	1						G	TT										
G12	1		A	A							T							
G13	1		A	A														
G14	1		A		A				T			A			T			G
G15	1		A											A			T	G
G16	1	T			A				T			A			T			G
G17	1			A									A		T			
G18	1			A							T							

Table 2: Details of mutations observed at different positions in the amplified 271bp region of the promoter, attenuator and coding regions of the *ampC* gene. Each genotype is shown, with the corresponding numbers of strains allocated to that group. Genotype numbers were allocated within this study. Position numbers for locations on the *ampC* gene were those used by Jaurin *et al.* (1981).[12] The control sequence is derived from the GenBank entry for *E. coli* NCTC 12241 (AY899338).

ins = 1 or 2 bp insertions at position -13.